Oxygen and Hydrogen Isotope Fractionation during Cellulose Metabolism in \textit{Lemna gibba} L.\(^1\) \\
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ABSTRACT \\

\textit{Lemna gibba} L. B3 was grown under heterotrophic, photoheterotrophic, and autotrophic conditions in water having a variety of hydrogen and oxygen isotopic compositions. The slopes of the linear regression lines between the isotopic composition of water and leaf cellulose indicated that under the three growth conditions about 40, 70, and 100\% of oxygens and carbon-bound hydrogens of cellulose exchanged with those of water prior to cellulose formation. Using the equations of the linear relationships, we estimated the overall fractionation factors between water and the exchanged oxygen and carbon-bound-hydrogen of cellulose. At least two very different isotope effects must determine the hydrogen isotopic composition of \textit{Lemna} cellulose. One reflects the photosynthetic reduction of NADP, while the second reflects exchange reactions that occur subsequent to NADP reduction. Oxygen isotopic composition of cellulose apparently is determined by a single type of exchange reaction with water. Under different growth conditions, variations in metabolic fluxes affect the hydrogen isotopic composition of cellulose by influencing the extent to which the two isotope effects mentioned above are recorded. The oxygen isotopic composition of cellulose is not affected by such changes in growth conditions.

The use of natural variations in the ratios of stable isotopes in plant biology has grown in recent years (24). Although carbon isotopes have been the main focus of attention (22), oxygen and hydrogen isotopes have much to offer. The differences in isotopic composition between ground water and leaf water, caused by the effects of transpiration on leaf water, are the basis for the potential use of stable isotopes in plant water relations (15, 29). Moreover, the isotopic composition of leaf water is recorded in leaf organic matter (5, 12, 16, 18, 20). An integrated isotopic signal that reflects the average growing conditions of the plant, with respect to temperature, humidity and water availability, may therefore be obtained.

Leaf water is the source for all organically bound hydrogen and is also the major factor influencing the oxygen isotopic composition of organic matter (9). In both cases there are large isotopic fractions in going from leaf water to organic matter (10, 14, 28). It is essential that the relationships between the isotopic composition of leaf water and organic matter be thoroughly understood to interpret isotopic ratios of organic matter. To date, only a limited number of studies directly addressed this question (14, 28). In these studies either oxygen or hydrogen isotopes were investigated. A combined isotopic study of the two elements would be very useful. This is because oxygen and hydrogen isotopic ratios of water, the ultimate source of plant isotopic signals, are highly correlated with each other (12, 26), while the additional oxygen and hydrogen isotope effects associated with biochemistry are not (14, 28). Thus, changes in oxygen and hydrogen isotopic composition of organic matter that are correlated may indicate source effects while noncorrelated changes would indicate additional biochemical effects.

Isotopic fractionations between water and cellulose, the organic fraction that is typically analyzed, can be expressed:

\[ \alpha_{\text{cellulose-water}} = R_{\text{cellulose}} / R_{\text{water}} \]

which \( R \) denotes the ratio between the heavy and the light isotopes, \textit{i.e.} \(^{18}\text{O}/^{16}\text{O} \) or \( D/H \). These ratios, in cellulose or water samples, are usually reported with reference to a standard, in this case SMOW\(^4\), using the \( \delta \) notation,

\[ \delta X = \left[ \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right] \times 1000\% \]

where \( X \) is \(^{18}\text{O} \) or \( D \). Combining the \( \alpha \) and \( \delta \) notations one can obtain:

\[ \delta_{\text{cellulose}} = \alpha_{\text{cellulose-water}} \times \delta_{\text{water}} + \left( \alpha_{\text{cellulose-water}} - 1 \right) \times 1000\% \]

Because \( \alpha \) values are close to unity for the processes involved in cellulose synthesis (\textit{e.g.} typically 1.00 \pm 0.10), Equation 3 can be simplified, for a first approximation, to:

\[ \delta_{\text{cellulose}} = \delta_{\text{water}} + F \]

where \( F \), the difference between the isotopic composition of cellulose and water, is an approximation of the deviation of \( \alpha \) from unity in \( \% \). \( F \) and \( \alpha \) can be related by the approximation \( F = 1000 \ln \alpha \).

If there is an additional source besides water whose oxygen or hydrogen is incorporated directly into cellulose without

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\(^4\)Abbreviations: SMOW, standard mean ocean water; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; RPP, reductive pentose phosphate; TP, triose phosphates.
exchanging with water, Equation 3 must be modified to:

$$\delta_{\text{cellulose}} = n(\delta_{\text{water}} + F) + (1 - n)\delta_Y$$  \hspace{1cm} (5)

where \(n\) is the fraction of hydrogen or oxygen that is influenced by water and \(Y\) is the additional source. It is important to emphasize that an intramolecular isotopic homogeneity for oxygen and hydrogen in \(Y\), as well as in cellulose, must be assumed because of present limitations imposed by the analytical procedures; i.e., we cannot measure isotope ratios for individual positions within molecules.

Equation 5 can be rearranged to obtain:

$$\delta_{\text{cellulose}} = n \times \delta_{\text{water}} + [n \times F + (1 - n)\delta_Y]$$  \hspace{1cm} (6)

In this study we grew \textit{Lemma} in water with a variety of isotopic compositions and determined the corresponding isotope ratios of leaf cellulose and that of any external source for oxygen or hydrogen. This enabled us to use the relationships described in Equation 6 to obtain information on the oxygen and hydrogen fractionation factors \((F)\) between water and cellulose associated with biochemical processes. By growing the \textit{Lemma} autotrophically, heterotrophically, or photoheterotrophically we could relate the fractionation factors obtained in each case to the metabolic pathways active under the specific growth conditions.

**MATERIALS AND METHODS**

**Plant Materials**

\textit{Lemma gibba} L. B3 was grown at 27°C. For each of the experiments described here, 9 to 15 \textit{Lemma} plants at the two-frond stage were transferred to a 3 L flask containing 500 mL of \(E\) media (7). \(E\) media without sucrose, or \(E\) media plus kinetin (2.5 \(\mu\)M) for photoheterotrophic, autotrophic, and heterotrophic growth conditions, respectively. The media was prepared with tap water to which various amounts (1-3 mL) of water enriched in \(^{18}\)O and deuterium (Isotec Inc. Miamisburg, OH) were added to obtain ranges in the oxygen and hydrogen isotopic composition of the water.

Heterotrophically grown plants were kept in complete darkness except for 2 min of weak red light every 8 h. For the other growth conditions, continuous light from a mixture of white fluorescent and incandescent light (about 200 \(\mu\)E m\(^{-2}\) s\(^{-1}\)) was used. For the photoautotrophic conditions the flasks were tightly closed, whereas for the autotrophic conditions a stream of dry compressed air was introduced into the flasks through a 9 mm Pyrex tube inserted in the rubber stopper. The air flow rate was increased gradually during the first 2 weeks of each experiment from 10 to a maximum of 40 mL min\(^{-1}\). This was found to supply the minimum flow rate necessary to not limit growth. The level of moisture and the CO\(_2\) concentration of the input air were tested using a portable gas exchange system (LiCor 6000, LiCor, Lincoln, NE). Concentration of CO\(_2\) for the autotrophic experiments was 330 \(\mu\)L L\(^{-1}\), except for one experiment in which a special mixture of air containing 1500 \(\mu\)L L\(^{-1}\) CO\(_2\) was used. A 40 cm long Pyrex tube served as an air cooled condenser on the outlet of every flask. High relative humidity, assumed to be close to 100\%, was indicated by the heavy condensation inside the flasks and along the first 1 to 2 cm of the condenser. The level of the media in each flask was marked in the beginning of the experiment; the change at the end of the experiment was negligible. The experiments were terminated (after about 2 weeks when sucrose was supplied in the media and about 3 weeks with no sucrose) when the media surface was fully covered with \textit{Lemma} leaves (several thousands fronds). It is an important feature of the experimental setup that at the end of each experiment the contribution of the small plant inoculum to the harvested biomass was negligible. Upon termination of each experiment, the \textit{Lemma} plants were quickly filtered and dried with a paper towel before a sample of 2 g was sealed in a test tube for vacuum distillation of leaf water (11). The rest of the plants were used for cellulose (10) or starch (2) extractions.

**Determination of Isotopic Composition**

A 0.5 mL sample of the vacuum distilled leaf water was equilibrated with CO\(_2\) at 25°C. An aliquot of the CO\(_2\) was cryogenically purified under vacuum and was used for the mass spectrometer analysis as described before (11). A second subsample of 5 \(\mu\)L was used to determine the hydrogen isotopic composition. The sample was sealed in a capillary which was broken under vacuum and the water was passed over uranium at 750°C to form, quantitatively, uranium oxide and release molecular hydrogen (4). The hydrogen was expanded into a sample tube and used for the mass spectrometric analysis.

The carbon isotopic composition of organic samples (cellulose, sucrose, etc.) was determined by heating them in evacuated sealed quartz tubes in the presence of copper, copper oxide, and silver at 875°C; then purifying the CO\(_2\) formed during combustion cryogenically prior to analyzing it mass spectrometrically. The oxygen isotopic composition of organic samples was analyzed after heating (525°C) about 6 mg in an evacuated tube with HgCl\(_2\) to obtain a mixture of CO\(_2\), CO, and HCl. The HCl was removed by passing the gas over liquid isoquinoline. The CO was disproportionated to CO\(_3\) and C by high voltage discharge and the combined CO\(_2\) fractions were collected for mass spectrometer analysis as described before (5, 23). Approximately 50 mg of each cellulose sample was nitrated by the nitric acid:acetic anhydride method (8) to remove hydroxyl hydrogen that may exchange with water during the preparative procedure. A sample (about 10 mg) of the cellulose nitrate, which contained only carbon-bound nonexchangeable hydrogen, was sealed under vacuum with copper, copper oxide and silver and was heated at 520°C. The water formed was passed over uranium as described above to produce molecular hydrogen, which was used for the mass spectrometer analysis.

Oxygen isotopic composition of air O\(_2\) was determined by quantitatively converting it to CO\(_2\). About 15 mL of air sample was introduced into an evacuated analysis system. After freezing out water and CO\(_2\) the air was circulated, by means of a mercury Toepler pump, over red hot graphite (about 2 g) in a small platinum vessel for 25 min. The CO\(_2\) thus produced was collected cryogenically and used for the mass spectrometer analysis.

All mass spectrometer analysis was performed on a MAT-250 mass spectrometer and \(\delta\) values (see introduction for
RESULTS AND DISCUSSION

Heterotrophic Growth Conditions

Sucrose in the media is the only carbon source when plants are grown in complete darkness. Under these completely heterotrophic growth conditions, the observed slope of the linear regression line between the isotope ratios of cellulose and water was about 0.4 for both oxygen and hydrogen (Fig. 1, A and D). These linear relationships can be interpreted with Equation 6 when we use for δ7 the isotope composition of sucrose (δ7O = +33.0‰, δ7D = −41‰). The slope indicates that about 40‰ of oxygen and of carbon-bound hydrogen have exchanged with and have had their isotopic composition influenced by water prior to cellulose formation. This estimate for oxygen is in agreement with that previously made with carrot cell culture (28).

From Equation 6 and using the γ-intercepts obtained from Figure 1, A and D and the isotopic composition of sucrose reported above, we calculated the overall fractionation factors, F1, between water and the exchanged oxygen or carbon-bound hydrogen in cellulose formed in the dark. The values obtained for F1 were +26.1‰ and +158‰ for oxygen and hydrogen respectively. The value for oxygen is about 10‰ higher than that reported previously for heterotrophically grown carrot tissue culture (28), but identical to that obtained in the same study when glycerol, rather than sucrose, was used as the carbon source. The relationship between hydrogen isotopic composition of water and that of the total organic matter of algae grown in the dark has been studied (13, 14). It was suggested that under these conditions, the organically bound hydrogen assumed the isotopic composition of the exogenously supplied sugar, i.e. no exchange with water occurred. This was clearly not the case in the present study.

Photoheterotrophic Growth Conditions

When Lemna plants were grown in closed vessels with sucrose in the media as before but under continuous light, the slope of the cellulose/water line was significantly higher than that observed in the dark (Fig. 1, B and E). Under these conditions the leaves were green and had a functional photosynthetic apparatus that was, most likely, operating at the compensation point (1). At least part of the sucrose taken in by the plants was metabolized through the RPP pathway under these conditions, i.e. while fixing respired CO2. As can be expected, this leads to a further exchange with water of both oxygen and hydrogen originating from sucrose. The slopes of about 0.7 for the linear regression lines between the isotope ratios of cellulose and water (Fig. 1, B and E) indicate that now about 70‰ of the oxygen and carbon-bound hydrogen of cellulose underwent exchange with water prior to cellulose formation. Because there was no external source for CO2, or hydrogen, the remaining 30‰ must have come directly from sucrose with no exchange with water. (The lack

Figure 1. Relations between leaf water and cellulose oxygen (A, B, C) and hydrogen (D, E, F) isotopic compositions in Lemna gibba grown under heterotrophic (A, D) and photoheterotrophic (B, E) conditions with sucrose in the media as carbon source and under autotrophic conditions with normal dry air (C, F). The correlation coefficients for the linear regression lines are better than 0.9 in all cases.
of external CO₂ contribution was confirmed by the δ¹³C value of cellulose, -10.6‰, similar to that of the cane sugar, -11.4‰, provided in the media. In contrast, cellulose from autotrophically grown Lemma had a δ¹³C value of -23.6‰.

Under photoheterotrophic growth conditions, some exchange occurs in the light that did not occur when Lemma was grown heterotrophically in the dark. The oxygen or hydrogen isotopic composition of cellulose grown photoheterotrophically can be described as follows:

\[ \delta_{\text{cellulose}} = p(\delta_{\text{water}} + F2) + q(\delta_{\text{water}} + F1) + [1 - (p + q)]6\delta \]  

(7)

where \( p \) is the fraction of oxygen or carbon-bound hydrogen that exchanged with or derived from water respectively during metabolic steps taking part in the chloroplast that are engaged only in the light, \( q \) is the fraction that exchanged with or derived from water during metabolic steps independent of chloroplast activity that are engaged both in the dark and in the light, and \( \delta \) is the isotopic composition of the exogenously supplied sucrose. This treatment involves a few assumptions. First, the pathways operating in the dark, and the corresponding exchange reactions with water, also operate in the light and to the same extent. Second, the fractionation factor \( F1 \) between cellulose and water for these sections of the pathway remains the same in the dark and in the light. Third, any intermediate metabolized via the RPP pathway, the pathway engaged in the light, had the opportunity to exchange all its oxygen and hydrogen with water.

Equation 7 can again be rearranged to describe the linear relationship observed between the isotopic ratios of water and cellulose:

\[ \delta_{\text{cellulose}} = (p + q)\delta_{\text{water}} + [p \cdot F2 + q \cdot F1 + [1 - (p + q)]6\delta] \]  

(8)

Equation 8 reduces to Equation 6 when \( p = 0 \) (\( q = n \)). We assume that the proportion of oxygen, or hydrogen, that was incorporated directly from sucrose into cellulose and the proportion of oxygen or hydrogen that originated from sucrose but underwent exchange with water via the ‘dark reactions’ was the same in the dark and in the light (i.e. \( q/[1 - (p + q)] \) = constant). We can, therefore, estimate \( q \) by comparing the ratios \( n/(1 - n) \) from the heterotrophic experiment (i.e. Eq. 6) and \( q/[1 - (p + q)] \) from the photoheterotrophic experiment (i.e. Eq. 8). Doing so, we obtain 0.24 and 0.21 for \( q \) (and consequently 0.47 and 0.44 for \( p \)) based on the results for oxygen or hydrogen isotopic composition respectively (Fig. 1, A and B and 1, D and E, respectively). Using these estimates of \( p \) and \( q \) in Equation 8 to interpret the linear regression lines shown in Figure 1, B and E, we obtain F2 values of +33‰ and +27.2‰ for hydrogen and oxygen respectively.

As indicated above, we assumed that the intermediates of the photosynthetic reactions that occurred only in the light-grown plants have exchanged or derived all their oxygen or hydrogens, respectively, with water. It is clear, however, that some of these must have been reexchanged as they passed through the ‘dark reactions,’ i.e. the same section of the pathway reflected also in the heterotrophic conditions. F2, therefore, represents a composite of F1, for the reexchange via the ‘dark reactions,’ and F3, the fractionation factor associated only with that section of the pathway that was active in the light. Thus:

\[ F2 = r \cdot F1 + (1 - r)F3 \]  

(9)

We now treat the sugars formed autotrophically in the same way as we did the exogenously supplied sucrose under the heterotrophic conditions, as sugars from both sources are incorporated into cellulose in a similar way. Therefore, we can obtain an estimate of F3 by substituting the fraction of oxygen or hydrogen exchanged with water under heterotrophic growth conditions for \( r \) in Equation 9. Doing this calculation, we obtain F3 values of -69‰ and +27.9‰ for hydrogen and oxygen, respectively.

Undoubtedly, the fractionation factors given above do not reflect isotope effects during a single reaction (Fig. 2). In fact, each reflects the integration of isotope effects of complete sections of pathways. In the dark, F1 may reflect exchange that occurs as glucose is polymerized directly into cellulose, i.e. due to F6P–G6P isomerization, together with the exchange that occurs as some sugars are completely metabolized as they enter the respiratory pathway. In the light, the situation is more complex. The high starch content observed in the leaves from the photoheterotrophic conditions indicated that substantial amounts of the exogenously supplied sugar was metabolized to TP. These compounds could enter the chloroplast, the site of starch accumulation, where part of them could be involved in various steps of the RPP pathway, including that of hydrogen donation by NADPH, before either exiting the chloroplast or being converted to glucose and polymerized to starch. There are several steps along this route that allow both hydrogen and oxygen exchange with water. Interestingly, both in the dark and in the light almost
all the steps allowing hydrogen (19) and oxygen exchange with water involve ketose-aldose isomerization.

The fractionation factors obtained for oxygen, F1, F2, and F3 were all within the range +26% to +28%. These values are consistent with the direct observation of oxygen isotope fractionation occurring during carbonyl hydration (fractionation factor +27%, ref. 27) and also support the hypothesis that under physiological conditions this reaction is the only way oxygen may exchange with water (27; see also 25). The only difference among the calculated fractionation factors, with regard to oxygen, is that each represents different sections of pathways, or metabolic fluxes, that allow different proportions of oxygens to exchange with water.

With regard to hydrogen, the results indicate that a strong discrimination against D is associated with photosynthetic activity. This is in agreement with an observation previously made in algae (13, 14). The reactions during which NADP is reduced by protons from water and, consequently, hydrogen is donated by NADPH during photosynthesis, are the only likely candidates for this effect. This is because all other reactions that could allow hydrogen exchange with water can occur under heterotrophic conditions as well. However, since NADPH formation involves an irreversible step and, in addition, practically all NADPH is consumed during well balanced photosynthetic activity, it is most likely that the negative isotope effect is associated only with the reduction of NADP with protons from water.

Clearly, the isotope effect associated with NADPH was only partially expressed in F3 because F3 represents various additional steps that allow the exchange of hydrogen with water, *i.e.* in the RPP pathway. Little is known about the proportions of the various metabolic fluxes involved in carbohydrate metabolism under photoheterotrophic conditions. Hydrogen stable isotope analysis, however, may prove to be an ideal tool to study these fluxes, once the specific fractionation factors involved are determined.

Interestingly, the oxygen isotopic composition of cellulose was similar to that obtained for starch from the same leaves (Table I). As mentioned above, under photoheterotrophic conditions, starch was formed in the chloroplasts from exogenous carbohydrates (17). The results, therefore, indicate that both starch and cellulose are metabolized from a common, well mixed, pool of TP and there are no oxygen isotope effects associated with the specific steps leading thereafter to either product.

| Table I. δ¹⁸O Values of Water, Cellulose, and Starch from Leaves of L. gibba Grown Photoheterotrophically with Sucrose in the Media |
|------------------|------------------|------------------|
| Water            | Cellulose        | Starch           |
| δ¹⁸O             |                  |                  |
| −14.5            | +20.7            | +22.7            |
| +25.5            | +45.6            | +47.3            |
| +42.2            | +56.8            | +55.7            |
| +62.0            | +69.7            | +67.2            |
| +81.8            | +83.3            | +81.5            |

Autotrophic Growth Conditions

When *Lemna* plants were grown under continuous light in media containing only mineral salts with a flow of dry air, the slope of the linear regression line between the isotopic composition of cellulose and water was approximately 0.8 for both oxygen and hydrogen (Fig. 1, C and F).

It is important to distinguish at this point between the isotope effects associated with the biochemical processes, which are the main subject of this study, and source effects on the isotopic ratios of cellulose. As discussed above, water, under autotrophic conditions, determines both the hydrogen and oxygen isotopic composition of plant organic matter. Biochemical isotope effects must remain constant as plants are grown in water with different isotopic compositions but under otherwise identical conditions. Under these circumstances the slope of the relationship between the isotopic ratios of cellulose and water should be one. If this is the case, direct estimate of F could be obtained from Equation 4. The results presented in Figure 1, C and F, with slopes of about 0.8, did not produce the expected relationships. In the next segment, we argue that these lower slopes are due to effects of atmospheric moisture that entered the experimental system. We then make the appropriate corrections and proceed to obtain an estimate of the fractionation factor, F, between cellulose and water as mentioned above.

Initially, we tested and rejected two possible hypotheses that could explain a slope of less than one for the relationship between the oxygen isotopic ratios of cellulose and water. The first possibility was the incorporation of an isotopic signal from air O₂. This possibility, which is all but ruled out by the experiments reported by Berry *et al.* (3), was eliminated in the present study. When *Lemna* plants were grown in air containing oxygen with a δ¹⁸O value of 377 ‰, the δ¹⁸O of leaf cellulose was +21.5 ‰, similar to the value of +20.9 ‰ obtained for control plants, for which the δ¹⁸O of the air O₂ was +23.6 ‰. Although atmospheric oxygen must be fixed into glycolate during photorespiration (3), this isotopic signal is lost due to partial release as CO₂ and isotopic exchange of the oxygen derived from O₂ with water before the products of photorespiration are incorporated into sugars. We also tested the possibility that some oxygen from air CO₂ is incorporated into cellulose without exchanging with water. If this were the case, growing *Lemna* with higher concentrations of CO₂ in the air should increase the proportion of nonexchanged oxygen incorporated into cellulose, thereby lowering the slope of the cellulose–water δ¹⁸O relationship. Growing *Lemna* with air containing 1500 µL L⁻¹ CO₂, compared with 330 µL L⁻¹ measured in the normal dry air, produced a cellulose/water slope of 0.71 (Fig. 3, compare with a slope of 0.77 in Fig. 1C). This is not a substantial shift for a CO₂ concentration five times higher than the control. We therefore concluded that isotopic signals from neither atmospheric O₂ nor atmospheric CO₂ were incorporated into cellulose.

The most striking point in the results shown in Fig. 1, C and F is the similarity in the slopes obtained for oxygen and for hydrogen. Water is not only the sole source for all organically bound hydrogen but also the only factor that could affect both the oxygen and hydrogen isotopic ratios simulta-
Figure 3. Relation between the oxygen isotopic composition of leaf water and that of cellulose in L. gibba grown with an air mixture containing 1500 μL L⁻¹ CO₂, under continuous light at 27°C. Correlation coefficient of the linear regression line is 0.995.

neously. We suspected, that water other than that provided in the media “contaminated” the experimental system. No moisture was present in the input air (see “Materials and Methods”). It is possible that an appreciable amount of moisture diffused back from the moist air in the growth chamber through the outlet lines leading to the atmosphere. To test this hypothesis, we compared the hydrogen isotopic ratios of the media and the condensed moisture, respectively, at the inner tip of the condenser on the outlet of a growth vessel. The δD values obtained were +54‰ for the media and +44‰ for the condensed moisture in one of the vessels used for autotrophic growth. The δD value of the atmospheric moisture in the lab was −95‰.

If the condensed atmospheric moisture is at equilibrium with the free vapor, as would be expected inside the condenser, its hydrogen isotopic ratio would be −95 + 77 = −18‰, since 77‰ is the equilibrium fractionation factor for hydrogen at 27°C (21).

Using this value we estimated the proportion of the atmospheric moisture contamination in the sample that was collected from the condenser, as follows:

\[ Z(-18‰) + (1 - Z)(+54‰) = +44‰ \] (10)

where Z represents the fraction of water in the condenser originating from atmospheric moisture, and −18‰, +54‰, and +44‰ are the δD values of the condensed moisture originating from the atmosphere, the media, and the vapor moisture at the exit from the growth vessel, respectively. A value of 0.14 is obtained for Z.

Using the value of 0.14 for Z and the isotopic composition of atmospheric moisture reported above, a corrected value for the relevant part of leaf water at equilibrium with the moisture in the growth vessel was calculated. The linear regression lines obtained for these corrected water isotope ratios and the cellulose isotope ratios reported in Figure 1, C and F were:

\[ \delta^{18}O_{\text{cellulose}} = 0.99 \delta^{18}O_{\text{water}} + 27.1‰ \quad r = 0.998 \] (11)

\[ \delta D_{\text{cellulose}} = 0.95 \delta D_{\text{water}} - 23‰ \quad r = 0.998 \] (12)

The intercepts obtained in Equations 11 and 12 can be interpreted using Equation 4 to give the overall fractionation factors between water and the exchanged oxygen or carbon-bound hydrogen in cellulose when all metabolites have passed through the RPP pathway. The intercept for oxygen in Equation 11, +27.1‰, is in good agreement with the estimates made above as well as with previous reports (Table II, 27). This, again, suggests that the same type of fractionation mechanism with the same fractionation factor is operating in all parts of the pathway leading to cellulose. With regard to hydrogen, the intercept in Equation 12, −23‰, must, like F2 above, represent a composite of F3, for the reactions associated with photosynthesis only, and F1, for the subsequent reexchange, estimated as +158‰, from the heterotrophic growth experiment (Table II). The results of the heterotrophic experiment provided us also, as discussed above, with a first approximation of the proportion of the reexchange that occurs after sugars are formed, i.e. about 40 to 45% of the oxygen and carbon-bound hydrogen. We can now calculate again the reexchange factor for F3 using Equation 9, substituting −23‰ for F2, 0.45 for r and +158‰ for F1. We obtain for autotrophic growth F3 = −171‰ (see Table II for a summary of the F values). Again, a very strong negative isotope effect associated with photosynthetic activity is indicated. The value obtained here, however, is much more negative than that obtained under photoheterotrophic conditions (F3 = −69‰). This difference may reflect differences in the proportions of the metabolic fluxes involved in carbohydrate metabolism under the different growth conditions. For example, under photoheterotrophic conditions (CO₂ at the compensation point, normal O₂ and light), the photorespiratory flux would be relatively large (6). Under these conditions the effects of hydrogen donation from NADPH, the likely source of the large negative isotope effect, would be less pronounced since the opportunity for hydrogen reexchange with water during the photorespiratory pathway would be greater. Under autotrophic conditions, on the other hand, the flux through the RPP pathway would increase, with no comparable change in the photorespiratory flux (6). Thus the NADPH effect would be preserved in the final product, such as cellulose, to a greater extent. An alternative explanation follows from the observation that a large starch accumulation was observed under photoheterotrophic but not under autotrophic conditions. Starch metabolism may increase the opportunity for hydrogen

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Table II. Overall Fractionation Factors between Water and Cellulose for Various Sections of Pathways Leading to the Synthesis of Cellulose under Different Growth Conditions

The values for F1 and F2 were calculated based on the linear regression lines reported in Figure 1. F2 was assumed to be a combination of F1 and F3. F1 was assumed to remain constant under all conditions.
to reexchange with water subsequent to the RPP pathway and before incorporation into cellulose. In any case, it appears that F3 obtained under the autotrophic conditions (−171‰) provides a better estimate of the net isotope effect associated with NADPH formation. Undoubtedly, more work is needed to confirm the hypotheses suggested here. But, at the same time, the potential in using stable isotopes in studying metabolic fluxes under different physiological conditions, once the fractionation factors are known, should be obvious.

Support for our hypothesis that there is a single oxygen isotope effect and two major hydrogen isotope effects in going from water to cellulose comes from a recently advanced argument regarding the relationships between the isotope ratios of oxygen and hydrogen in water and leaf matter (26). Plotting the isotope ratios of hydrogen versus those of oxygen for either leaf water or leaf cellulose yields linear relationships (Fig. 4), (21, 24, 26). It was demonstrated (26) that the equation of the δD versus δ18O line of leaf cellulose can be related to that of leaf water by:

\[
\delta D_{\text{cellulose}} = s(\alpha_D/\alpha_{18})\delta^{18}O_{\text{water}} + \left[1000(\alpha_D - 1) - c - 1000\times s(\alpha_D/\alpha_{18})(\alpha_{18} - 1)\right] \quad (13)
\]

where \(s\) is the slope of the water line, \(\alpha_D\) and \(\alpha_{18}\) are the hydrogen and oxygen fractionation factors, respectively, between cellulose and water and \(c\) is the intercept of the water line. Equation 13 describes a linear relationship in which the slope of the cellulose line, \(s(\alpha_D/\alpha_{18})\), is a function of the slope of the leaf water line, \(s\), and the ratio between the hydrogen and oxygen fractionation factors. Since we propose that as we change from heterotrophic to autotrophic growth conditions, \(\alpha_{18}\) remains constant but \(\alpha_D\) changes dramatically, one would expect that the ratio \(\alpha_D/\alpha_{18}\), and therefore the slope of the cellulose line relative to that of the water line, would also change accordingly. We used the slopes of the water lines reported in Figure 4, together with the values of \(\alpha_D\) (1.71, 0.977) and \(\alpha_{18}\) (1.027, 1.027) for heterotrophic and autotrophic conditions, respectively (derived from the F values reported above, see also introduction), to calculate the predicted slopes of the corresponding cellulose lines. We obtain values of 1.69 and 1.51 for the slopes of the cellulose lines under heterotrophic and autotrophic conditions, respectively (i.e. 1.69 = [1.171/1.027]).48 and 1.51 = [0.977/1.027]).59. These values are in agreement with the actual slopes obtained (Fig. 4). Thus, not only can we accurately predict changes in slopes in the cellulose relative to the water lines as growth conditions change, but such changes may serve as an indicator of the metabolic pathway used by the plant in each case.

Obviously more work is needed to identify the specific biochemical steps responsible for the observed isotope effects and to determine the fractionation factors involved. This information is essential to understanding the variations in the stable oxygen and hydrogen isotopic composition of plant matter. This understanding, in turn, can help in providing new insights into changes in metabolic fluxes involved in plant metabolism under different growth conditions.

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