Short-Term Measurement of Carbon Isotope Fractionation in Plants

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ABSTRACT

Combustion-based studies of the carbon-13 content of plants give only an integrated, long-term value for the isotope fractionation associated with photosynthesis. A method is described here which permits determination of this isotope fractionation in 2 to 3 hours. To accomplish this, the plant is enclosed in a glass chamber, and the quantity and isotopic content of the CO₂ remaining in the atmosphere are monitored during photosynthesis. Isotope fractionation studies by this method give results consistent with what is expected from combustion studies of C₃, C₄, and Crassulacean acid metabolism plants. This method will make possible a variety of new studies of environmental and species effects in carbon isotope fractionation.

Studies begun in the 1950s demonstrated that δ¹³C values for plants are more negative than that of atmospheric CO₂; that is, plants contain less ¹³C than does atmospheric CO₂ (1, 5, 6, 16, 21, 22). Subsequently, it was shown that there is a systematic difference between C₃ plants (δ¹³C near −27‰/oo) and C₄ plants (δ¹³C near −13‰) (2, 3, 24). Within these two broad classes, environmental and species effects are small (16). Delta values for CAM plants are more variable because they reflect the two available photosynthetic modes: Nocturnal CO₂ fixation introduces carbon with a δ¹³C value near −11‰, whereas daytime CO₂ fixation introduces carbon with a δ¹³C value near −27‰/oo (14, 19, 20). Environmental variations which change the CAM/C₃ balance change the δ¹³C value accordingly.

Throughout the history of this field, investigators have hoped that isotopic compositions might be useful in studying environmental and species effects. However, except in the case of CAM plants, this has not proved to be the case. Although several thousand species of terrestrial plants have been subjected to isotopic analysis, interspecies variations are small (16). Neither light intensity (21, 26) nor temperature (23, 25–27) produces a substantial change in δ¹³C. Combustion analysis of 120 strains of Zea mays failed to identify any strains which differed significantly from the mean (MH O'Leary, DW Weber, unpublished data). In only a few cases have significant variations been seen: Isotopic compositions of halophytes vary with salinity (10, 15).

Isotopic compositions of various strains of wheat correlate with water use efficiency (9). Isotopic compositions of C₄ plants vary slightly with bundle sheath permeability (11).

Measurement of δ¹³C values of plant materials has generally been carried out by combustion of dried leaves or other plant parts. The isotopic composition so obtained is a long-term integration of environmental and developmental effects. Early in leaf development, carbon is imported from elsewhere in the plant, and this carbon contributes to the isotopic signal. In a mature leaf, carbon is incorporated as a result of local photosynthesis, but some of this carbon is exported to elsewhere in the plant. As senescence approaches, the export rate increases and the photosynthetic rate decreases. All these phenomena contribute to the isotopic composition obtained in combustion analysis. This long-term integration undoubtedly masks a number of effects and is probably responsible for the lack of environmental and species variations cited above.

A more detailed isotopic signal can probably only be obtained by short-term studies in which the isotopic composition (or fractionation) is measured over a period of a few hours. In the case of CAM plants, we have established a method for studying the isotope fractionation associated with nocturnal CO₂ fixation that makes use of the isotopic composition of carbon-4 of newly formed malate (17). This isotopic information reflects the history of the plant during a short term and is more nearly independent of long-term influences. Studies have been made of the effect of temperature (8), CO₂ concentration (12), and species (17) on the isotope fractionation associated with nocturnal CO₂ fixation. A similar short-term approach to C₃ and C₄ plants should reveal short-term variations in isotope fractionation which are not currently measurable. For a variety of reasons, methods analogous to the malate method are unlikely to be successful. We describe in this paper a general method in which the change in isotopic composition of atmospheric CO₂ is monitored during photosynthesis in a closed compartment. A similar method based on gas-exchange has been used by Berry (JA Berry, personal communication).

MATERIALS AND METHODS

Plants. All plants were grown in the University of Wisconsin Biotron with a 10 h day. The night temperature was 17°C and the day temperature was 23°C. Plants were watered daily with half-strength Hoagland solution. Experiments were conducted 2 to 4 h after the lights were turned on for C₃ and C₄ plants. For CAM plants, nocturnal fixation studies were conducted 2 to 4 h after the lights were turned on, and daytime fixation studies were conducted 6 to 9 h after the lights were turned on.

Soybean (Glycine max) was strain Mitchell. The two youngest triplets of fully expanded leaves were used in each experiment. Zea mays was strain W64A. The youngest fully expanded leaf was used for isotope fractionation studies. Kalanchoe daigremontiana was from the same clone used in previous studies in our

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2 The definition of δ¹³C is

\[ \delta^{13}C = (R_{sample}/R_{standard} - 1) \times 1000 \]

where \( R \) is the ratio ¹³CO₂/¹²CO₂ derived from the mass spectrometer measurements. A more positive value of δ¹³C means that the sample contains more ¹³C. Isotope fractionation = δ¹³Csample − δ¹³Cproduct.
CARBON ISOTOPE FRACTIONATION

laboratory (8). The eight youngest fully expanded leaves were
used.

Isotope Fractionation Experiment. The reaction chamber con-
stituted of a metal base plate sealed to an 12-L glass bell jar. The
plate was divided in half so that the stem of the plant could be
introduced through the center. The plate had inlet and outlet
ports for pumping air through the chamber. The amount of leaf
was chosen so that at least 75% of the initial CO₂ was taken up
in about 1 h.

The petiole of the plant in question was sealed with Apiezon
Q between two halves of the plate, and then the two halves were
clampped together and sealed with wax. The plate was supported
on a ring stand. Then the bell jar was placed over the leaves and
sealed to the plate with stopcock grease. Air was pumped through
the bell jar at a flow rate of 4.5 L/min for 15 min. (It is important
that the air intake be placed near the air intake to the room, so
that effects of CO₂ from experimenters' breath is minimized).
Stopcocks on the entrance and exit ports were closed and the air
in the bell jar was sampled by means of an evacuated 1-L flask
equipped with a vacuum stopcock. Entrance and exit stopcocks
were reopened, and air was flushed through the chamber for a
further 15 min, after which the stopcocks were closed again.
After a 5-min wait, the air in the bell jar was sampled again by
the same procedure. The bell jar was then flushed with air for a
further 15 min, and the system was sealed again. After an
appropriate wait, a further sample was taken. This flush-wait
procedure was repeated as many times as desired in order to
accumulate the desired set of time points. The time reported for
each point (and shown in the Figures) is the time after sealing.
Exposure times were generally at 5 min intervals. For a 40-min
exposure time, more than half of the initial CO₂ was invariably
gone.

Isotopic Analyses. The CO₂ samples so collected were purified
on a high-vacuum line equipped with a diffusion pump. Sample
sizes were calculated by use of a calibrated manometer or, for
small samples, from the pressure when the sample was admitted
to the inlet of the mass spectrometer. Volumes of sample flasks
were accurately known. The bell jar system was tight enough
that sampling reduced the pressure in the system by approxi-
mately one-twelfth and this was taken into account in calculating
CO₂ concentration. Final sizes of CO₂ samples were 2 to 15
µmol.

Isotopic compositions were measured on a Finnigan Delta-E
isotope-ratio mass spectrometer and were corrected for instru-
mental effects (7). Replicate analyses of a single sample could
be accomplished with a reproducibility of ±0.05‰ or better.
Delta values are reported relative to the usual PDB standard (7).

Combustions were conducted in sealed, evacuated quartz tubes
in the presence of 0.5 g of CuO wire which had previously been
heated to 875°C for 2 h. Leaf material was introduced in a Ag
boat. After 2 h at 875°C, the temperature was reduced to less
than 550°C and the heating was continued for a further 12 h.

THEORY

If a plant is taking up CO₂ in an open atmosphere, then the
isotopic difference between leaf and atmosphere corresponds to
the isotope fractionation

\[
\text{CO}_2 \text{(atm)} \rightarrow \text{leaf}
\]

\[
\delta^{13} \text{C (CO}_2\text{)} - \delta^{13} \text{C (leaf)}
\]

In the absence of industrial activity the \(\delta^{13} \text{C}\) value for atmo-
spheric CO₂ is relatively constant at about -8‰ (17), so com-
bustion analysis of a leaf can be used to obtain the isotope
fractionation.

In a closed container, the atmosphere gradually becomes de-
pleted in CO₂ and a more complex mathematical treatment is
necessary. Because of the discrimination against \(^{13}\text{C}\) in the pho-
tosynthetic process, the atmosphere becomes slightly enriched in
\(^{13}\text{C}\). As this happens, the isotopic content of newly introduced
carbon also changes, even though the isotope fractionation re-
mains constant. Experiments of this type have long been used in
chemical systems, and equations are available which permit

calculation of the isotope fractionation from the change in iso-
topic content of either the source or the product (4).

This same approach can be used for obtaining the isotope
fractionation associated with CO₂ uptake in plants, provided that
the time- and concentration-dependence of CO₂ uptake are
known. At reasonably high light, the CO₂ absorbance rate is
roughly proportional to the CO₂ concentration. In addition, CO₂
is given off by plants (respiration) in a process that is probably
independent of CO₂ concentration. Taken together, these two
processes give, for the CO₂ atmosphere,

\[
d(CO₂)/dt = -k_1 \left[CO₂\right] + k_2
\]

where \([CO₂]\) is the concentration of CO₂ at any time, \(k_1\) is the
first-order rate constant for CO₂ uptake, and \(k_2\) is the zero order
rate constant for CO₂ release by respiratory processes. Rate
constant \(k_2\) is calculated by use of the fact that at the compensa-
tion point, \(k_2 = k_1 \left[CO₂\right]\). When appropriate initial conditions
are included and the equation is integrated, the result is

\[
[CO₂] = ([CO₂]_{\text{initial}} - [CO₂]_{\text{comp}}) e^{-k_1 t} + [CO₂]_{\text{comp}}
\]

where \([CO₂]_{\text{initial}}\) represents the concentration at the beginning of
the experiment and \([CO₂]_{\text{comp}}\) represents the concentration at the
compensation point. Separate expressions of this form can be
written for \(^{12}\text{CO}_2\) and \(^{13}\text{CO}_2\), the difference being that \(k_1\) for
the two isotopes differs by the isotope fractionation associated with
CO₂ uptake (this is the quantity of interest) and relative values
of \(k_2\) reflect the isotopic content of respired carbon.

It should be noted that the way the equations are written
specifically includes the possibility that respired CO₂ which is
released into the chamber may be refixed. Second, this treatment
recognizes that the isotopic composition in the chamber is chang-
ing.

Combining separate equations of the type given above permits
us to predict the change in \(\delta^{13} \text{C}\) value of atmospheric CO₂ which
occurs during photosynthesis. The variables are

(a) \(\delta^{13} \text{C}\) of atmospheric CO₂ at the beginning of the experi-
ment
(b) the isotope fractionation associated with CO₂ uptake
(c) the rate of CO₂ uptake
(d) the rate of formation and \(\delta^{13} \text{C}\) of respired carbon

The experimental data were fitted by first using the measured
CO₂ concentration versus time to give values for \(k_1\) and \(k_2\). In
the case of CO₂ uptake, special care was taken to obtain an optimum
value of \(k_2\) based on the changes in isotopic composition late in
the experiment. Once these values were derived, the isotope
fractionation associated with \(k_1\) was varied until the best fit to
the experimental data was obtained. The isotopic content of
respired carbon was assumed to be the same as that of whole
leaves. This value has only a minor effect on the derived param-
eters.

RESULTS

Two triplets of mature leaves from a soybean plant were placed
in a closed 12 L container in the light, and the atmosphere was
sampled at 5 or 10 min intervals for 45 min. During this period,
the CO₂ concentration decreased to about 15% of its initial value
and the isotopic composition of the CO₂ in the container showed
a significant change (Fig. 1).

During the first part of the experiment, the \(\delta^{13} \text{C}\) value became
quite positive (residual CO₂ became enriched in \(^{13}\text{C}\)) as the plant
discriminated against \(^{13}\text{C}\) during photosynthesis. Late in the
experiment, the rate of photosynthesis became quite small and the isotopic content was principally influenced by respiratory processes. The trend toward more negative $\delta^{13}$C values late in the experiment was observed consistently. The isotopic composition of respiratory carbon is expected to be much more negative than the atmosphere (perhaps near -27‰; [16]), so the $\delta^{13}$C value again became more negative. During this period there was little change in CO$_2$ concentration. Fitting of the isotopic data as described under "Theory" produced an isotope fractionation of 20‰. Atmospheric CO$_2$ in the Biotron is -8‰, so this isotope fractionation would be expected to produce a leaf $\delta^{13}$C value of -28‰, if the short-term isotope fractionation is the same as the long-term one. The $\delta^{13}$C value obtained by combustion of the same leaf was -27.0‰.

Repetition of this same experiment on the next day resulted in the same pattern of CO$_2$ uptake (data not shown) and gave an isotope fractionation of 20‰. Repetition of the experiment with a different set of leaves under the same environmental conditions resulted in a somewhat different CO$_2$ absorption rate (on an absolute basis) but a corresponding difference in the pattern of isotopic changes. The scatter in the data was somewhat larger than in the previous experiments, and the calculated isotope fractionation was 17‰.

Similar experiments on a mature leaf of maize showed a similar pattern of CO$_2$ uptake, except that the CO$_2$ concentration approached zero at the end of the experiment (Fig. 2; other data not shown). The change in isotopic composition of environmental CO$_2$ over the course of the experiment was much smaller than with soybean, consistent with the expectation of a smaller isotope fractionation in this case, and there was no tendency of $\delta^{13}$C values to become more negative late in the experiment. Data fitting by the same procedure gave an isotope fractionation of 4.5‰. If this short-term isotope fractionation is the same as that occurring over the whole life of the leaf, then combustion analysis should produce a $\delta^{13}$C value of -12.5‰. The observed value was -12.2‰.

CAM plants such as K. daigremontiana fix CO$_2$ at night by a C$_4$-like pathway and sometimes fix CO$_2$ in the late afternoon by the direct C$_3$ pathway (13, 18). The course of the change in isotopic composition during nocturnal CO$_2$ fixation is similar to that seen in C$_4$ plants (Fig. 3). The calculated isotope fractionation was 7‰, which would give rise to a whole leaf $\delta^{13}$C value of -15‰. When K. daigremontiana is exposed to CO$_2$ only during the night, the combustion $\delta^{13}$C value has been reported to be -11‰ (14). Afternoon CO$_2$ uptake (3 PM) by the C$_3$ pathway in K. daigremontiana (Fig. 4) gave an isotope fractionation of 19‰, which would give rise to a whole leaf $\delta^{13}$C value of -26‰, similar to the value of -27‰ seen in K. daigremontiana exposed to CO$_2$ only during the day (14). Evidently these plants are engaging in both CAM (Fig. 3) and daytime (Fig. 4) CO$_2$ fixation.

**DISCUSSION**

Qualitatively, the isotopic composition data show the expected pattern: there is a large change in the $\delta^{13}$C value of remaining CO$_2$ in the case of C$_3$ photosynthesis, and only a small change in remaining CO$_2$ during C$_4$ photosynthesis. The isotopic pattern of nocturnal CO$_2$ fixation in the CAM plant K. daigremontiana parallels that of C$_4$ photosynthesis. The isotopic pattern for daytime fixation in the same species parallels C$_3$ photosynthesis. More quantitatively, the isotope fractionations calculated here are consistent with those expected for C$_3$ and C$_4$ photosynthesis, both based on combustion analysis of the leaves actually used in these experiments and on the body of experience regarding combustion-based isotope fractionations for a variety of C$_3$ and C$_4$ plants (16).

In general, we do not expect an exact correspondence between the results of the short-term studies and the results of combustion analyses. As noted previously, isotopic compositions obtained by combustion analysis contain information reflecting the entire history of the leaf, whereas the short-term method reflects only a short period. The fact that the short-term method is independent of any isotopic anomalies occurring during development is...
of particular importance.

Photosynthetic rates respond to CO2 concentrations, and it is possible that the isotope fractionation might change in the course of these short-term experiments because of changes in stomatal aperture, carboxylation capacity, or other factors. However, the maximum exposure of the leaf to low concentrations of CO2 is only for a period of about 15 min, and we do not expect that photosynthetic capacity would change enough in that period to perturb the isotopic results. In future experiments, we will collect more data in the early part of the CO2 uptake curve, thus eliminating this problem.

Respiration is an important confounding variable in studies with C3 plants. The quantity of respired carbon is small, but its isotopic composition is very different from that of the atmosphere (16). Early in the experiment, the fraction of atmospheric carbon that is derived from respiration is small, but the $\delta^{13}C$ value of that respired carbon is quite different from that of the atmospheric CO2. Later in the experiment, when more CO2 has been taken up, the proportion of atmospheric carbon that is derived from respiration increases. The mathematical treatment takes account of the fact that some refixation of this respired CO2 occurs, but both the respiration rate and the isotopic composition of the CO2 thus produced have an important influence on the derived isotope fractionation. The isotopic compositions late in the experiment (after $\delta^{13}C$ has begun to become more negative) provide a satisfactory basis on which to estimate the respiration correction. In future experiments, the isotopic composition of atmospheric CO2 will be adjusted to be approximately the same as that of the atmosphere, thus making the respiration correction much smaller.

The method described here for studying isotope fractionation in plants is capable of producing short-term isotope fractionations with a precision of ±1%, and this can probably be improved in future experiments. Studies of nocturnal CO2 fixation in CAM plants have shown that short-term experiments provide a wealth of detail that is not available in combustion studies. This method should be particularly useful for study of, e.g., developmental effects, environmental effects, and a number of other factors that may affect photosynthetic rates and efficiencies.

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LITERATURE CITED

6. CRAIG H 1954 Carbon 13 in plants and the relationships between carbon 13 and carbon 14 variations in nature. J Geol 62: 115-149

