Plant Physiol. (1997) 114: 391–394

Rapid Communication

Carbon Isotopic Fractionation Does Not Occur during Dark Respiration in C_3 and C_4 Plants[1]

Guanghui Lin[2]* and James R. Ehleringer

Stable Isotope Ratio Facility for Environmental Research, Department of Biology, University of Utah, Salt Lake City, Utah 84112

The magnitude of possible carbon isotopic fractionation during dark respiration was investigated with isolated mesophyll cells from mature leaves of common bean (*Phaseolus vulgaris* L.), a C_3 plant, and corn (*Zea mays* L.), a C_4 plant. Mesophyll protoplasts were extracted from greenhouse-grown leaves and incubated in culture solutions containing different carbohydrate substrates (fructose, glucose, and sucrose) with known $\delta^{13}C$ values. The CO_2 produced by protoplasts after incubation in the dark was collected, purified, and analyzed for its carbon isotope ratio. From observations of the isotope ratios of the substrate and respired CO_2, we calculated the carbon isotope discrimination associated with metabolism of each of these substrates. In eight of the 10 treatment combinations, the carbon isotope ratio discrimination was not significantly different from 0. In the remaining two treatment combinations, the carbon isotope ratio discrimination was 1‰. From these results, we conclude that there is no significant carbon isotopic discrimination during mitochondrial dark respiration when fructase, glucose, or sucrose are used as respiratory substrates.

In all higher plants organically bound carbon in leaves is depleted in ^{13}C relative to the carbon of atmospheric CO_2. This depletion results from isotopic discrimination events associated with CO_2 fixation into the leaf, including (a) CO_2 diffusion through leaf stomata and membranes, (b) carboxylation by Rubisco, and (c) carboxylation by PEP carboxylase (Farquhar et al., 1989). Dark respiration has usually been dismissed as a minor component, even though few data are available to evaluate the possible magnitude of any isotopic fractionation during mitochondrial respiration (O'Leary, 1981; Farquhar et al., 1982, 1989; Schleser and Jayasekera, 1985; Schleser, 1990).

At the whole-tissue level Baertschi (1953) observed no significant difference in carbon isotope ratios between bean seedlings and whole bean seeds, which led him to conclude that there was no carbon isotopic fractionation during plant respiration. In follow-up studies Park and Epstein (1961) and Jacobson et al. (1970) showed that the isotopic composition of respired CO_2 could change significantly over several hours, implying possible fractionation during respiration. However, additional fractionation events that occur during metabolism, including fractionation during pyruvate decarboxylation (DeNiro and Epstein, 1977; Melzer and Schmidt, 1987), will broadly influence the isotopic composition of major biochemical groups such as lipids and terpenes. Thus, if there are any temporal patterns to normal maintenance respiration, this could account for the nocturnal variations in the ^{13}C content of leaf respiration. On the other hand, Troughton et al. (1974) found differences in carbon isotope ratios between respired CO_2 and whole-plant tissues, with carbon isotope ratios being 5‰ higher than organic material for the respired CO_2 than whole-plant materials in wheat (*Triticum aestivum*) and 4‰ lower in monterey pine (*Pinus radiata*). Based on these studies and on calculations of the fluxes of carbon within leaves, Farquhar et al. (1982) concluded that any carbon isotope fractionation associated with plant respiration would likely be negligible. Little more concerning this topic has been investigated, except that Henderson et al. (1992) speculated at the end of their recent study that the observed small discrepancies between leaf carbon isotope discrimination of C_4 plants and the same values measured using on-line discrimination measurements could be associated with a fractionation event associated with respiration.

In this study we investigated the magnitude of carbon fractionation during mitochondrial respiration in mesophyll cells isolated from mature leaves of both a C_3 and a C_4 species to directly test the hypothesis that there is no significant carbon isotope fractionation during mitochondrial dark respiration.

MATERIALS AND METHODS

Plant Material and Preparation of Intact Mesophyll Protoplasts

Common dry bean (*Phaseolus vulgaris* L. var San Juan Select) and corn (*Zea mays* L. var Incredible hybrid) were grown in pots under greenhouse conditions at the University of Utah (Salt Lake City). Intact mesophyll protoplasts

[1] This study was supported by a grant from the Office of Health and Research at the U.S. Department of Energy.

[2] Present address: Research Department, Biosphere 2 Center, P.O. Box 689, Oracle, AZ 85623, and Lamont Doherty Earth Observatory of Columbia University, Palisades, NY 10964–8000.

* Corresponding author; e-mail glin@bio2.edu; fax 1–520–896–6214.

Abbreviation: Δ_m, carbon isotope discrimination during dark respiration.

were isolated from approximately 10 g of fresh leaf material (mature leaves from 5- to 6-week-old plants), which had been kept in the dark for 2 d prior to experiments to reduce background carbohydrate levels. The abaxial and adaxial epidermis were carefully stripped from each leaf with forceps, and then leaves were cut into 10- × 5-mm^2 strips. The strips were placed in a preplasmolysis medium containing 0.3 M mannitol and 1 mM $CaCl_2$ for 30 min and then transferred into a Petri dish with 40 mL of an enzyme medium containing 0.4 M mannitol, 1 mM $CaCl_2$, 0.25% (w/v) BSA, 10 mM sodium ascorbate, 2% (w/v) cellulase Onozuke R-10, and 0.2% (w/v) Macerozyme R-10 (Sigma) in 10 mM Mes-KOH, pH 5.5 (Devi et al., 1992). After 30 to 40 min, the digestion medium was gently removed from the dishes and several milliliters of a resuspension medium containing 0.4 mM mannitol and 1 mM $CaCl_2$ in 10 mM Mes-KOH (pH 6.0) was added to the dish. The protoplast suspension was passed successively through 300-, 80-, and 60-μm nylon filter cloths to remove undigested leaf portions. The filtrate was then centrifuged at 50*g* for 10 min, after which the pellet was washed with a fresh batch of the resuspension medium and the protoplast pellet was collected after centrifugation again at 50*g* for 5 min. The final protoplasts were diluted in 1 mL of the above-mentioned suspension solution.

Incubation of Mesophyll Protoplasts with Fru, Glc, and Suc

Dry, CO_2-free air was added to 10-mL pre-evacuated containers (Vacutainers, Becton Dickinson) using a vacuum line and each container was filled to atmospheric pressure. A 1-mL medium containing 0.4 mM mannitol, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Hepes-KOH, pH 7.6, was added to the container using a gas-tight syringe. Then, depending on the experiment, 50 mmol of Fru, Glc, or Suc (C_4-signal materials from Sigma and C_3-signal materials from Professor E.-D. Schulze, Universität Bayreuth, Germany) was added to the reaction container. Following this, 0.5 mL of protoplast suspension was added to the reaction medium in each container. The pH of the final mixture was 6.9 ± 0.1 (n = 6). The containers were then placed into a water bath at 25°C in the dark for different incubation periods (24, 48, and 72 h). Following the incubation, each container was removed from the water bath and immediately reconnected to a vacuum line to extract and purify the CO_2 produced during the incubation period (using an alcohol-dry ice slush trap to remove water, followed by a liquid nitrogen trap to collect the CO_2; Ehleringer and Osmond, 1989). Corrections were made for the minor respiratory consumption of mannitol in the preparation medium by measuring the CO_2 production and its isotopic composition in control suspensions receiving no Fru, Glc, or Suc. The amount of CO_2 produced in each container during an experiment was measured with a digital manometer (Cole-Parmer, Chicago, IL). There were three to four independent replications per treatment and per time interval.

To examine the possible effect of medium pH on the carbon isotope ratio of respired CO_2, we conducted a separate series of experiments in which protoplasts were incubated with 50 mmol of Fru at different pH values for 48 h. The pH values of different incubation mixtures (n = 3 per treatment) were adjusted to between 3 and 10 by adding either 1 N NaOH or 1 N HCl into the reaction mixture using a gas-tight syringe. After the CO_2 had been collected from these incubation containers, the incubation pH values of the final solution were again measured.

Carbon Isotope Analyses of Respired CO_2, Substrate, and Leaves

Carbon isotope ratios of purified CO_2 were measured in an isotope-ratio mass spectrometer (Delta S, Finnigan MAT, Bremen, Germany) in the dual-inlet mode. The carbon isotope ratios of leaf materials and carbohydrate substrates (Fru, Glc, and Suc) were analyzed on the same mass spectrometer using an elemental analyzer to combust the organic material and generate CO_2.

The carbon isotope ratio was expressed in δ notation as:

$$\delta^{13}C(‰) = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000‰,$$

where R is the molar ratio of $^{13}C/^{12}C$ of the sample and standard (Pee Dee belemnite). The overall precision for carbon isotope analysis including combustion and gas transfer was ±0.11‰, based on repeated analyses of a laboratory standard (Utah cabbage).

Δ_m was defined as:

$$\Delta_m = \frac{\delta^{13}C_{substrate} - \delta^{13}C_{CO_2}}{1 + \delta^{13}C_{substrate}},$$

where $\delta^{13}C_{substrate}$ and $\delta^{13}C_{CO_2}$ are the carbon isotope ratios of the substrate and the evolved CO_2 gas, respectively.

RESULTS AND DISCUSSION

The respiratory substrates used in this study were from commercially available carbohydrates with carbon isotope ratios typical of C_3 and C_4 plants (Table I). Fully expanded leaves from greenhouse-grown common bean (a C_3 plant) had $\delta^{13}C$ values of −28.3 ± 0.4‰ (n = 10), and those from greenhouse-grown corn (a C_4 plant) had a $\delta^{13}C$ value of −11.7 ± 0.1‰ (n = 11), which were significantly different

Table I. *Carbon isotope ratio values of leaves and carbohydrate sources used in this study*

Data are means ± 1 SD.

Substrate	n	$\delta^{13}C$
		‰
Bean leaves	10	−28.3 ± 0.4
Corn leaves	11	−11.7 ± 0.1
C_3 Suc	4	−25.1 ± 0.1
C_4 Suc	4	−10.0 ± 0.1
C_3 Glc	4	−25.8 ± 0.1
C_4 Glc	4	−9.5 ± 0.1
C_4 Fru	4	−10.1 ± 0.1

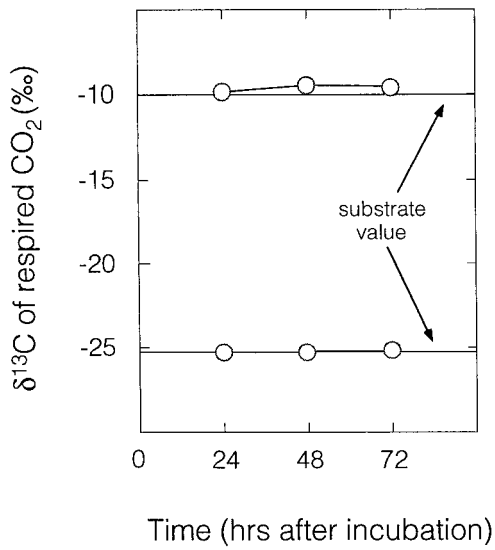

Figure 1. Time course of the carbon isotope ratio of the respired CO_2 from corn mesophyll protoplasts after incubation with C_3- and C_4-derived Suc at 25°C and at a pH value of 6.9.

from the respiratory substrates used here ($P < 0.001$ for all comparisons between the leaf samples and the substrates [Tukey's test]). Thus, any CO_2 released by mesophyll protoplasts could be distinguished from the respired CO_2 from consumption of any respiratory substrate.

The carbon isotope ratio of CO_2 evolved from protoplasts under the different treatments did not change over the 24- to 72-h intervals following the addition of the substrate (Fig. 1). Thus, the calculated Δ_m values were constant through time, and each of the individual observations were therefore pooled to increase sample sizes for the statistical analyses.

Of the 10 possible combinations of C_3/C_4 substrate source and plant material, 8 of the calculated carbon isotope discrimination values were not significantly different from $\Delta_m = 0$ (Table II). These results were expected based on previous discussions by O'Leary (1981) and Farquhar et al. (1982), who both predicted that dark respiratory fractionation would be negligible. In none of the treatments in which C_3 or C_4 protoplasts were fed C_3 sugars was there any indication of any significant carbon isotope fractionation during respiration. However, two of the six C_4-sugar treatments did exhibit a small negative discrimination during respiration (Table II). The reason for these exceptions are not clear but may have been due to experimental variance. Overall, however, our results suggest that Δ_m was negligible.

Table II. Δ_m *of Suc, Glc, and Fru protoplasts of bean and corn leaves*

Data are means ± 1 SD, with the sample size shown in parentheses.

Substrate	Discrimination by Bean Protoplasts	Discrimination by Corn Protoplasts
	‰	
C_3 Suc	−0.1 ± 0.2 (12)	0.2 ± 0.2 (12)
C_4 Suc	−0.6 ± 0.8 (9)	−1.4 ± 0.2 (9)[a]
C_3 Glu	0.3 ± 0.2 (12)	0.0 ± 0.3 (12)
C_4 Glu	−1.2 ± 0.3 (12)[a]	−0.0 ± 0.4 (12)
C_4 Fru	0.6 ± 0.8 (12)	−0.1 ± 0.3 (12)

[a] Significantly different from 0 at the $P < 0.05$ level.

In a closed system there will be no carbon isotope discrimination if all of the substrate is consumed. In this study the total amount of substrate carbon added a as respiratory substrate in each vacuum container was 300 μmol of carbon when Fru and Glc were used and 600 μmol when Suc was used. The maximum total amount of CO_2 produced by the end of the sampling period with Fru and Glc substrates was 140 μmol of CO_2, which was only 47% of the total original substrate. The maximum total amount of CO_2 produced by the end of the sampling period with Suc as a substrate was 210 μmol of CO_2, which was only 35% of the total original substrate. Thus, we conclude that the lack of significant carbon isotope discrimination was not the result of excessive substrate consumption.

One possible complication with our approach is that the pH of the incubation solution could significantly affect the $\delta^{13}C$ value of respired CO_2, since there is a significant isotopic fractionation between gaseous CO_2 and aqueous HCO_3, and pH plays a significant role in determining the relative proportion of these two components in solution (Deuser and Degens, 1967). We tested this possibility by varying the pH of the incubation solution and found that with pH values between 3.0 and 8.0, the carbon isotope ratios of respired CO_2 were still not significantly different from that of the substrate (Fig. 2). At pH values greater than 8, the $\delta^{13}C$ value of the respired CO_2 was slightly different from that of the sugar substrate. Therefore, because the pH of the incubation solution used in this study was 6.9 ± 0.1, the pH should not have had a significant impact on the interpretation of our results.

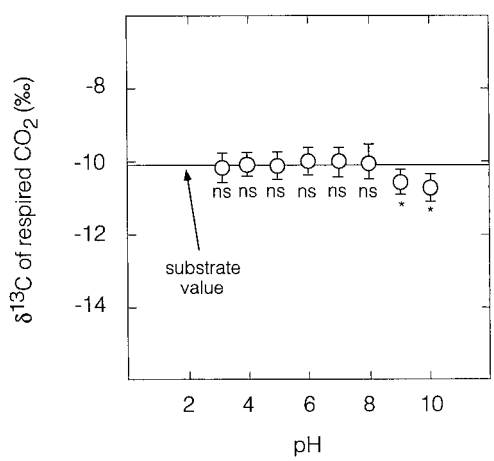

Figure 2. Carbon isotope ratio (mean ± 1 SD, $n = 3$) of the respired CO_2 from corn mesophyll protoplasts after incubation with Fru in a closed system for 48 h at 25°C, with the pH of the final incubation medium adjusted at the end of incubation. The solid line represents the mean carbon isotope ratio of the Fru substrate. The slope of the regression is not significantly different from 0. The intercept of the regression is not significantly different from the source substrate value.

Physiological interpretations of the leaf carbon isotope ratios in C_3 and C_4 plants hinge on models linking carbon isotope fractionation events and physiological parameters such as the leaf intercellular CO_2 concentration (Farquhar et al., 1989). An implicit assumption of these models is that respiratory fractionation events are nonexistent or so small as to be negligible. Since as much as one-half of the daily carbon gain by a leaf might be respired, quantification of possible mitochondrial respiratory fractionation events is critical for physiological interpretations of whole-leaf carbon isotope ratios.

Our results support current working models proposing that carbon isotope discrimination during mitochondrial respiration does not have a significant influence on determining variations in leaf carbon isotopic composition. This confirms the approach taken by Farquhar et al. (1982, 1989), Farquhar and Richards (1984), and Farquhar and Lloyd (1993), in which (a) variations in the intercellular CO_2 concentration and diffusion through the stomata account for the majority of the observed differences in leaf isotope composition, and (b) respiratory discrimination effects are hypothesized as being sufficiently low so as to be negligible in modeling efforts. Our results have implications for interpreting physiological and ecological studies in which only whole-leaf isotopic composition is measured, since these studies assume that there is no respiratory fractionation.

Carbon isotope ratios of respired CO_2 have been used to indicate shifts in respiratory substrates (Jacobson et al., 1970; Hsu and Smith, 1972; Walker et al., 1983; Tang et al., 1987) without verification that there was no fractionation associated with dark respiration. Our results suggest that carbon isotopic analysis of dark-respired CO_2 does indeed precisely indicate the substrate used during respiration and that previous studies by Park and Epstein (1961) and Jacobson et al. (1970) may have been observing time-dependent changes in the primary metabolites used for respiration. If this is indeed the case, then future nocturnal respiration studies may be able to distinguish between phloem-loading, maintnance, and construction-respiration activities within different cells and organs.

ACKNOWLEDGMENTS

We thank Yuan Ke, Susan Phillips, and C.F. Kitty for assistance. We also thank two anonymous reviewers for their suggestions regarding improvement of the manuscript.

Received September 27, 1996; accepted January 24, 1997.

LITERATURE CITED

Baertschi P (1953) Die Fractionierung der natürlichen Kohlenstoffisotopen im Kohlendioxydstoffwechsel grüner Pflanzen. Helv Chim Acta **36:** 773–781

DeNiro MJ, Epstein S (1977) Mechanisms of carbon isotope fractionation associated with lipid synthesis. Science **197:** 261–263

Deuser WG, Degens ET (1967) Carbon isotope fractionation in the system CO_2 (gas)-CO_2(aqueous)-HCO_3^-(aqueous). Nature **215:** 1033–1035

Devi MT, Vani T, Reddy MM, Raghavendra AS (1992) Rapid isolation of mesophyll and guard cell protoplasts from pea and maize leaves. Ind J Exp Biol **30:** 424–428

Ehleringer JR, Osmond CB (1989) Stable Isotopes. *In* RW Pearcy, JR Ehleringer, HA Mooney, PW Rundel, eds, Plant Physiological Ecology: Field Instrumentation and Methods. Chapman & Hall, New York, pp 281–300

Farquhar GD, Ehleringer JR, Hubick KT (1989) Carbon discrimination and photosynthesis. Annu Rev Plant Physiol Plant Mol Biol **40:** 503–537

Farquhar GD, Lloyd J (1993) Carbon and oxygen isotope effects in the exchange of carbon dioxide between terrestrial plants and the atmosphere. *In* JR Ehleringer, AE Hall, GD Farquhar, eds, Stable Isotopes and Plant Carbon-Water Relations. Academic Press, San Diego, CA, pp 47–70

Farquhar GD, O'Leary MH, Berry JA (1982) On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. Aust J Plant Physiol **9:** 121–137

Farquhar GD, Richards RA (1984) Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes. Aust J Plant Physiol **11:** 539–552

Henderson SA, von Caemmerer S, Farquhar GD (1992) Short-term measurements of carbon isotope discrimination in several C_4 species. Aust J Plant Physiol **19:** 263–285

Hsu JC, Smith BN (1972) $^{13}C/^{12}C$ ratios of carbon dioxide from peanut and sunflower seedlings and tobacco leaves in light and in darkness. Plant Cell Physiol **13:** 689–694

Jacobson BS, Smith BN, Epstein S, Latis GG (1970) The prevalence of carbon-13 in respiratory carbon dioxide as an indicator of the type of endogenous substrate. J Gen Physiol **55:** 1–17

Melzer E, Schmidt H-L (1987) Carbon isotope effects on the pyruvate dehydrogenase reaction and their importance for relative carbon-13 depletion in lipids. J Biol Chem **262:** 8158–8164

O'Leary MH (1981) Carbon isotope fractionation in plants. Phytochemistry **20:** 553–567

Park R, Epstein S (1961) Metabolic fractionation of C^{13} and C^{12} in plants. Plant Physiol **36:** 133–138

Schleser GH (1990) Investigation of the $\delta^{13}C$ pattern in leaves of *Fagus sylvatica* L. J Exp Bot **41:** 565–572

Schleser GH, Jayasekera R (1985) $\delta^{13}C$-variation of leaves in forests as an indication of reassimilation CO_2 from the soil. Oecologia **65:** 536–542

Tang W, Sternberg L, Price D (1987) Metabolic aspects of thermogenesis in male cones of five cycad species. Am J Bot **74:** 1555–1559

Troughton MJ, Card KA, Hendy CH (1974) Photosynthetic pathways and carbon isotope discrimination by plants. Carnegie Inst Washington Year Book **73:** 768–780

Walker DB, Gysi J, Sternberg L, DeNiro ML (1983) Direct respiration of lipids during heat production in the inflorescence of *Philodendron selloum*. Science **220:** 419–421