Carbon Isotope Discrimination measured Concomitantly with Gas Exchange to Investigate CO₂ Diffusion in Leaves of Higher Plants

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Abstract

Conventional gas-exchange techniques that measure the stomatal conductance and rate of CO₂ assimilation of leaves were combined with measurements of the carbon isotope composition of CO₂ in air passing over a leaf. Isotopic discrimination during uptake was determined from the difference in the carbon isotope composition of air entering and leaving the leaf chamber. Isotopic discrimination measured over the short term correlated strongly with that determined from combusted leaf material. Environmental conditions were manipulated to alter the relative influences of stomatal conductance and carboxylation on the discrimination of carbon isotopes by intact leaves. With C₃ plants, discrimination increased as the gradient in partial pressure of CO₂ across the stomata decreased. For C₄ plants there was little change in discrimination despite substantial changes in the diffusion gradient across the stomata. These results are consistent with, and provide the first direct experimental support for, theoretical equations describing discrimination during photosynthesis.

Despite uncertainties about various processes affecting carbon isotope composition, the resistance to the transfer of CO₂ from the intercellular airspaces to the sites of carboxylation in the mesophyll chloroplasts was estimated using this technique. For wheat the estimated resistance was 1.2-2.4 m² s bar mol⁻¹.

Introduction

The fractionation of the naturally occurring, stable isotopes of carbon (¹²C and ¹³C) in the process of photosynthetic CO₂ fixation is influenced by a number of biochemical and environmental factors (Vogel 1980; O’Leary 1981). A theoretical analysis, integrating the effect of diffusion and carboxylation in C₃ photosynthesis on discrimination has been developed (Vogel 1980; Farquhar 1980, 1983; Farquhar et al. 1982b; Farquhar and Richards 1984). The theory predicts that the net fractionation by C₃ plants can be described approximately by an equation having a diffusion-dependent term and a biochemistry-dependent term. This may be written as (Farquhar and Richards 1984, equation (2))

\[ \Delta = a \frac{P_a - P_i}{P_a} + b \frac{P_i}{P_a}, \]

where \( \Delta \) is the net discrimination against ¹³CO₂, \( P_a \) and \( P_i \) are the ambient and intercellular partial pressures of CO₂, \( P(CO₂) \), respectively, \( a \) is the fractionation occurring due to diffusion in air (4.4% = 4.4 × 10⁻³) and \( b \) is the net fractionation caused by...
RuP2* and PEP carboxylation (about 27%). The relative significance of these terms varies with $p_i$. If $p_i$ is near to $p_a$, the discrimination is dominated by the biochemical fractionation, whereas if $p_i$ is low relative to $p_a$, fractionation due to diffusion (the first term in equation (1)) dominates. Net discrimination is sensitive to the ratio of $p_i$ to $p_a$ because the magnitudes of $a$ and $b$ are quite different. Carbon isotope discrimination has generally been related to $p_i/p_a$ by rearranging equation (1) as

$$
\Delta = a + (b - a) p_i/p_a.
$$

(1a)

For C₄ plants, a simplified expression for discrimination is:

$$
\Delta = a \frac{p_a - p_i}{p_a} + (b_4 + b_3 \phi) \frac{p_i}{p_a} = a + (b_4 + b_3 \phi - a) p_i/p_a.
$$

(2a)

where $b_4$ is the net fractionation for CO₂ dissolving, hydrating to HCO₃⁻ and then being fixed by PEP carboxylase (-5.7%), $b_3$ is the fractionation caused by RuP₂ carboxylation (29%, Roeske and O'Leary 1984) and $\phi$ is the proportion of CO₂ fixed by PEP carboxylation which subsequently leaks back out of the bundle sheath (Farquhar 1983, equation (11)).

We have developed a new approach for measurement of carbon isotope discrimination over short time intervals in conjunction with measurements of leaf gas exchange. The method involves measuring the $^{13}\text{C}:{^{12}\text{C}}$ ratio of the CO₂ remaining after photosynthesis has consumed a portion of the CO₂ in the air. The primary aim of this work was to obtain direct experimental support for the theory relating carbon isotope discrimination to CO₂ assimilation in leaves. This was largely successful, as the net discrimination by C₃ leaves was linearly correlated with the ratio of $p_i$ to $p_a$, whereas for C₄ leaves the discrimination was nearly independent of this ratio. The second, more ambitious aim was to use an expression for $A$, more complete than equation (1), to estimate the resistance to CO₂ transport in the mesophyll.

The more complete description of discrimination by C₃ leaves which is approximated in equation (1) includes terms for gaseous diffusion of CO₂ through the leaf boundary layer and stomata, diffusion from the intercellular airspaces to the sites of carboxylation, enzymatic fixation of CO₂, by RuP₂ carboxylase and finally discrimination which may occur during respiration and photorespiration. The net discrimination can be written as:

$$
\Delta = a_b \frac{p_a - p_s}{p_a} + a \frac{p_s - p_i}{p_a} + (b_s + a_1) \frac{p_i - p_c}{p_a} + b \frac{p_c - p_a}{p_a} = \frac{e R_d/k}{1 + \Gamma} + \frac{f}{1 + \Gamma},
$$

(3)

where $p_a$ is the ambient partial pressure of CO₂, $p(CO₂)$, $p_s$ is the $p(CO₂)$ at the leaf surface, $p_i$ is the intercellular $p(CO₂)$, $p_c$ is the equivalent $p(CO₂)$ at the sites of carboxylation, $a_b$ is the fractionation occurring during diffusion in the boundary layer (2.9% = 2.9 $\times$ 10⁻³), $a$ is the fractionation due to diffusion in air (4.4%), $b_s$ is the fractionation occurring as CO₂ enters solution (1.1% at 25°C) (Vogel 1980), $a_1$ is the fractionation due to diffusion in water (0.7%), $b$ is the net fractionation caused by RuP₂ and PEP carboxylation (27%), $e$ and $f$ are fractionations with respect to average carbon composition associated with 'dark' respiration ($R_d$) and photorespiration, respectively, $k$ is the carboxylation efficiency (see Farquhar et al. 1982b, equation (B11)) and $\Gamma$ is the CO₂ compensation point in the absence of $R_d$. For the derivation and discussion of the fractionations see Farquhar et al. (1982b), equation (14), Farquhar (1983), equation (3), and Farquhar and Richards (1984), equation (A6).

The drop in $p(CO₂)$ between the intercellular spaces and the sites of carboxylation results from a finite CO₂ transfer resistance, $r$, such that

$$
p_i - p_c = Ar,
$$

(4)

* Abbreviations used: PAR, photosynthetically active radiation (400-700 nm); PEP, phosphoenolpyruvate; RuP₂, ribulose 1,5-bisphosphate.
where \( A \) is the rate of \( \text{CO}_2 \) assimilation. Combining equations (3) and (4), an expression for \( r \) is found:

\[
    r = \frac{\Delta_i - \left( eR_d/k + f \Gamma_a \right) / p_a - \Delta}{(b - b_s - a_1)A/p_a},
\]

(5)

where \( \Delta_i \) is the discrimination expected on the basis of equation (1), after allowing for boundary layer resistance, i.e.

\[
    \Delta_i = \frac{a_b}{p_a} - \frac{p_s - p_i}{p_a} + \frac{b}{p_a}.
\]

(6)

In principle, \( r \) can be estimated using equation (5) if the terms involving respiration and photorespiration are ignored. Alternatively, equation (5) may be rewritten to relate the deviation between \( \Delta_i \) and \( \Delta \) to \( A/p_a \),

\[
    \Delta_i - \Delta = r (b - b_s - a_1)A/p_a + (eR_d/k + f \Gamma_a/p_a).
\]

(7)

The slope of this relationship yields an estimate for \( r \) and the \( A/p_a \) intercept yields an estimate for the respiratory terms.

Theory

A formal expression relating the discrimination, \( \Delta \), of the leaf against uptake of \( ^{13}\text{CO}_2 \) to the ‘discrimination against \( ^{13}\text{CO}_2 \) in air moving past a leaf’, \( \Delta_a \), is derived in the Appendix as

\[
    \Delta = -\frac{\xi \Delta_a}{1 + \xi \Delta_a},
\]

(8)

where

\[
    \xi = \frac{c_e}{c_e - c_o}
\]

(9)

and \( c_e \) and \( c_o \) are the mole fractions of \( \text{CO}_2 \) in air, measured at a standard humidity, entering and leaving a well mixed chamber, respectively. \( \xi \) is the ratio of rate of \( \text{CO}_2 \) entry into the chamber to rate of net \( \text{CO}_2 \) fixation by the leaf. The leaf discriminates against \( ^{13}\text{CO}_2 \) (\( \Delta > 0 \)), which means that \( \Delta_a \) is negative, and the proportion of \( ^{13}\text{C} \) remaining in the air increases as the air is depleted of \( \text{CO}_2 \), i.e. as the flow rate is reduced (cf. equations (A7) and (A10) in Appendix). In practice, the isotope ratios of the air entering and leaving the chamber were not compared directly, but separately with respect to a standard, PDB. As shown in the Appendix, equation (3) then becomes

\[
    \Delta = \frac{\xi(\delta_o - \delta_e)}{1000 + \delta_o - \xi(\delta_o - \delta_e)}.
\]

(10)

where \( \delta_o \) and \( \delta_e \) represent the isotopic composition of \( \text{CO}_2 \) entering and leaving the chamber, respectively. Care was taken in the measurement of \( ^{13}\text{C}/^{12}\text{C} \) ratios (Mook and Grootes 1973) to allow for changes in the isotopic composition of oxygen, which occur when \( \text{CO}_2 \) passes over a leaf (K. Hubick, G. Farquhar and S. Wong, unpublished data).

Materials and Methods

Plant Material

Seeds of \( \text{Triticum aestivum} \) L. cv. Yecora 70, \( \text{T. monococcum} \) L. cv. Einkorn 292, \( \text{Xanthium strumarium} \) L., \( \text{Gossypium hirsutum} \) L. cv. Deltapine, \( \text{Phaseolus vulgaris} \) L. cv. Hawkesbury Wonder, \( \text{Zea mays} \) L. and \( \text{Amaranthus edulis} \) Speg. were sown in 5-litre pots filled with sterilized potting soil. Plants were grown in a well ventilated glasshouse with a 16-h photoperiod. The plants were watered twice daily and fertilized three times a week with a complete nutrient solution. Fully expanded leaves (flag or penultimate for wheat) were used for the gas exchange measurements and it was sometimes necessary to trim the leaf to fit it in the leaf chamber.
Gas Exchange

Compressed air was passed through columns of soda asbestos and magnesium perchlorate to remove CO₂ and H₂O. It was combined with sufficient 1% CO₂ in air, using two mass-flow controllers (Tylan FC 260; Torrance CA), to yield 340 μmol p(CO₂) before it entered the leaf chamber, where it was rapidly circulated with a fan (Micronel; Fallbrook CA). Two slide projectors with 150-W halogen lamps gave approximately 1000 μmol quanta PAR m⁻² s⁻¹ at the leaf surface. Leaf temperature was maintained at 23°C and was measured with a fine copper-constantan thermocouple (No. SCPSS-020E-6, Omega; Stamford CT) pressed against the lower surface of the leaf. Depletion of CO₂ by photosynthesis was sensed by a Binos 1 infrared gas analyser (Leybold-Heraeus, GmbH; Koln) and compensated by separate injection of the same 1% CO₂ directly into the chamber using another mass flow controller. To obtain measurably large values of Δ, it was necessary to reduce the total flow, u, as much as possible (cf. equations (A7) and (A11)). To avoid the consequent problem of humidification, a portion of the air from the chamber was circulated by a metal bellows pump through a trap surrounded by dry ice and ethanol. Humidity of the outflowing air was measured with a capacitative sensor (Vaisala, Finland) or an H₂O vapour sensing Binos 1. The gas exchange calculations included the corrections for evaporation (von Caemmerer and Farquhar 1981).

Carbon Isotope Composition

Air leaving the leaf chamber was dried by passing it through two H₂O-vapour traps (cooled by dry ice-ethanol) and the CO₂ collected in two U-tubes, in series, in liquid N₂. At the exit of the chamber the air line was connected by a tee-piece to a bubbler, to ensure that the pressure up to this point was greater than in the atmosphere. After this point the line was vacuum-tight and a gate valve regulated the air that was drawn through the traps by a rotary vacuum pump. The pressure at the CO₂-traps was less than 20 Torr (27 mbar), which was sufficiently low to avoid condensation of O₂.

When enough CO₂ had been collected (c. 50 μmol, which took 10-30 min, depending on the flow rate), the CO₂-traps were isolated from the gas exchange system and pumped down to < 10⁻³ Torr. The traps were then isolated from the pump and gently heated, allowing the sample to diffuse to a vial in liquid nitrogen. The sample was then transferred to the ratio mass spectrometer. Glass vials were found to be the most reliable when sealed in situ and broken in the mass spectrometer inlet. The CO₂ collection system was checked in several ways. The carbon isotope composition of the air was measured with reference to a Zea mays standard calibrated against primary limestone standards (Osmond et al. 1981), and found to be independent of the flow rate through the leaf chamber, and of the p(CO₂) in the air. Bubbling the air through water before entry into the vacuum line also did not affect the isotopic composition. Presumably the isotopic exchange equilibrated during the long times needed for several collections.

Generally the δ¹³C value of the air entering the chamber, which was obtained from a cylinder of pure CO₂, was -25.5%o. To examine the effect of the isotopic composition of the source, a special gas mixture was made. The CO₂ was generated from the reaction of finely ground shell (CaCO₃, δ¹³C = +2.3%o) with orthophosphoric acid (10 g CaCO₃ : 300 ml acid), under vacuum. The CO₂ was collected in U-tubes in liquid N₂ before being transferred to an evacuated gas cylinder. The isotopic composition was brought to -7.5%o by adding pure CO₂ (-25.5%o) and the CO₂ was then diluted with pure oxygen and nitrogen to yield 1% CO₂, 20% oxygen and 79% nitrogen.

Experimental Protocol

The gas exchange of the leaf was allowed to reach steady state under each experimental condition before CO₂ was collected. The rate of CO₂ assimilation and the stomatal conductance were measured before and after each collection. If significant drift had occurred, the sample was discarded. The precision of the method was improved considerably during the course of experiments and so the number of replicates was reduced. For C₃ plants, each datum is the mean of 3-10 replicates, while for C₄ plants each is a single determination. Variation in p₁/pₙ was obtained by manipulating light intensity or quality, p(O₂), or leaf-to-air vapour pressure difference. Some Phaseolus plants were grown in 150 mm NaCl and the Xanthium point with lowest p₁/pₙ was obtained with a detached leaf taking up 20 μM abscisic acid.

Results

Measurements of carbon isotope discrimination using the above technique were compared with values with discrimination calculated from the δ¹³C value of combusted leaf material (Fig. 1). For C₃ and C₄ species the short-term discrimination correlated strongly
Carbon Isotope Discrimination and CO₂ Diffusion

\( r^2 = 0.99 \) with integrated discrimination by the leaf. However, the short-term values were on average only 87% of those of whole leaf material. Since the new method is non-destructive, several samples can be collected from the same leaf under different conditions, which was useful in demonstrating the relationship between \( \frac{p_i}{p_a} \) and discrimination (Table 1). Various ratios of \( p_i \) and \( p_a \) were obtained by altering \( p(O_2) \), light quality and leaf-to-air vapour pressure difference. In general, the change in the observed discrimination agreed with the prediction from equation (1) for that change in the ratio of \( \frac{p_i}{p_a} \). In all cases the absolute value of discrimination for a given \( \frac{p_i}{p_a} \) was less than predicted. Changing the isotopic composition of the source CO₂ did not influence the net discrimination (Table 1).

![Graph showing short-term discrimination against ¹³CO₂ during photosynthesis with long-term discrimination](Image)

**Table 1.** Effect on net discrimination against ¹³CO₂ of changes in the ratio of intercellular to ambient \( p(CO_2) \) and of changes in the \( \delta^{13}C \) value of the source CO₂

<table>
<thead>
<tr>
<th>Species</th>
<th>Conditions</th>
<th>( \frac{p_i}{p_a} )</th>
<th>( \Delta_{\text{pred}} )</th>
<th>( \Delta_{\text{obs}} )</th>
<th>( n )</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Xanthium strumarium</em></td>
<td>21% O₂</td>
<td>0.84</td>
<td>23.4</td>
<td>21.7 ± 1.5</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>2% O₂</td>
<td>0.67</td>
<td>19.5</td>
<td>18.4 ± 0.2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(Difference)</td>
<td>- 3.9</td>
<td>- 3.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Triticum aestivum</em></td>
<td>White, 1250°A</td>
<td>0.77</td>
<td>21.8</td>
<td>15.9 ± 1.7</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Blue, 350°</td>
<td>0.92</td>
<td>25.2</td>
<td>22.2 ± 3.0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>(Difference)</td>
<td>+ 3.4</td>
<td>+ 6.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>T. aestivum</em></td>
<td>14 mbar vpdb</td>
<td>0.67</td>
<td>19.5</td>
<td>12.0 ± 0.2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>7 mbar</td>
<td>0.80</td>
<td>22.5</td>
<td>15.7 ± 3.2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(Difference)</td>
<td>+ 3.0</td>
<td>+ 3.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>T. aestivum</em></td>
<td>- 7.5%°C</td>
<td>0.84</td>
<td>23.4</td>
<td>18.4 ± 0.2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>- 25.5%°C</td>
<td>0.86</td>
<td>23.8</td>
<td>18.2 ± 0.2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>(Difference)</td>
<td>+ 0.4</td>
<td>- 0.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^A\) Irradiance (µmol quanta PAR m\(^{-2}\) s\(^{-1}\)). \(^B\) leaf-to-air vapour pressure difference. \(^C\) \( \delta^{13}C \) value of the CO₂ entering the chamber.
The short-term discrimination measured for several C₃ and C₄ species is shown as a function of the ratio of intercellular and ambient $p(CO_2)$ (Fig. 2). Considerable variation in discrimination was seen for the C₃ species and was positively correlated with $p_i/p_a$, as predicted by the theory. Discrimination by the C₄ species (Fig. 2) was virtually independent of $p_i/p_a$. The theoretical line for the C₃ plants is the simplified equation (1a) which has commonly been used and ignores the presence of boundary layer resistance, or any drop in the $p(CO_2)$ between the intercellular spaces and the sites of carboxylation, and of any effects associated with dark respiration or photorespiration (see equation (3)).

![Fig. 2. Relationship between observed discrimination against $^{13}CO_2$ and measured $p_i/p_a$. The simplified theoretical line for C₃ plants is: $\Delta = [4.4 + (27 - 4.4)p_i/p_a] \times 10^{-3}$. The simplified theoretical line for the C₄ plants is drawn assuming that the leakage, $\phi$, is 0.21 and 0.34 for Zea mays and Amaranthus edulis, respectively: $\Delta = [4.4 + (27\phi - 10.1)p_i/p_a] \times 10^{-3}$. Symbols as in Fig. 1. *Gossypium hirsutum.*](image_url)

![Fig. 3. The difference between discrimination predicted from equation (6) and that observed ($\Delta_i - \Delta_{obs}$) versus the rate of CO₂ assimilation, $A$, divided by the ambient $p(CO_2)$, $p_a$.](image_url)

The deviation of the observed discrimination from the line in Fig. 2 is plotted against $A/p_a$ in Fig. 3. According to equation (7), the slope of this relationship is proportional to the transfer resistance and the intercept is a measure of the fractionation associated with respiration and photorespiration. The scatter in the data reveal the limits imposed
by experimental error. Also, the treatments were designed to test the relationships between $\Delta$ and $p_i/p_a$ rather than between $\Delta$ and $A/p_a$. The open triangles represent measurements made with a carbon source of $-7.5\%$. These do not appear to differ from those obtained with a source of $-26$ to $-29\%$. The respiratory terms were ignored by constraining the regression of $\Delta_i - \Delta_{obs}$ against $A/p_a$ through the origin and the slope for T. aestivum was $61 \times 10^{-3}$ m$^2$s bar mol$^{-1}$.

Using equations (3) and (4) it was possible to make more accurate predictions of the discrimination by Triticum aestivum. We again assumed $b = 27\%$ and ignored the terms in equation (3) involving respiration, but included the effects of boundary layer resistance, and of the resistance to CO$_2$ transfer, the latter having been estimated independently as $2$ m$^2$s bar mol$^{-1}$ (Evans 1983a). The regression between $\Delta$ observed and $\Delta$ predicted from equation (3), constrained through the origin, had a slope of 0.98 ($r^2 = 0.66$, $P < 0.01$, data not shown) which demonstrates good absolute agreement between measurement and prediction.

Discussion

We have demonstrated that it is possible to measure the carbon isotope discrimination occurring during photosynthesis over a short time interval. Discrimination measured over the short term correlates very highly ($r = 0.99$) with the discrimination based on combusted leaf material. The results showed that carbon isotope discrimination is strongly influenced by the ratio of intercellular and ambient $p$(CO$_2$) as had been predicted (Farquhar et al. 1982b).

Indirect evidence in C$_3$ species supporting the relationship between discrimination and the ratio of intercellular to ambient $p$(CO$_2$) has previously been obtained by manipulating environmental conditions and comparing genotypes. Increased salinity (Guy et al. 1980; Farquhar et al. 1982a; Downton et al. 1985; Seemann and Critchley 1985) or decreased relative humidity (Winter et al. 1982), which are known to result in decreased stomatal conductance to CO$_2$ diffusion, have resulted in less discrimination as predicted. The flacca tomato mutant, which has abnormally high stomatal conductance, exhibits greater discrimination than the wild type (Bradford et al. 1983). The isotopic composition of wheat leaves correlated well with the water-use efficiency of the plants, which in turn is related to the ratio of $p_i/p_a$ (Farquhar and Richards 1984). Mistletoe plants, which tend to have higher conductances than their hosts, show greater discrimination than their hosts (Ehleringer et al. 1985). However, the isotopic composition of the plant material reflects an assimilation-weighted average of the discrimination occurring over the entire growth period and the effects of secondary metabolism and export of carbon compounds during the life of the leaf. Since it is not possible to keep $p_i/p_a$ constant, nor is it practicable to monitor $p_i/p_a$ over a long period of time, a critical test of the theory has been lacking. The present data represent the first direct link between gas exchange and isotopic fractionation. Considering the direct evidence reported here and the indirect evidence described above, the influence of $p_i/p_a$ on carbon isotope discrimination appears established.

The discrepancy between the data and equation (1) can probably be ascribed to the approximations made in obtaining equation (1) from the more complete equation (3). It seems unlikely that much of the deviation is due to effects of dark respiration continuing in the light. We compared the results obtained in normal air with that using air having a different isotopic composition, more like that of the leaf. If the effect of respiration rate (the term $eR_d/kp_a$) were significant in the light, it would be seen as a source of CO$_2$ assimilated during the measurement. While no significant difference was observed in the values of $\Delta$ in wheat for a change in $\delta_{air}$ of 18\% (Table 1, Fig. 3), this does not rule out the possibility since $R_d/kp_a$ is small in the light. With hindsight, the question could have been tested better by measuring the discrimination as a function of irradiance, as this would alter $A/p_a$ to a greater extent.
We have no estimate of the magnitude of the parameter \( f \), the discrimination against \( ^{13}\text{C} \) during photorespiratory decarboxylation of glycine. The magnitude of \( e \) itself is uncertain. Because it is a measure of the discrimination against substrate \( ^{13}\text{C} \) during respiration, and because respiration in the dark is important in the carbon balance of plants, a moderately negative value of \( e \) could explain the difference under discussion. Park and Epstein (1961) and Troughton et al. (1974) both found the \( ^{13}\text{C} \) value of carbon respired in the dark to be 5% more positive than the \( ^{13}\text{C} \) value of the leaf, which could mean that \( e \approx -5\% \). However, as discussed below, it may reflect the \( ^{13}\text{C}/^{12}\text{C} \) ratio of current photosynthate being greater than in structural carbon. Consistent with this, Hsu and Smith (1972) found the \( ^{13}\text{C} \) value of carbon respired in the dark to be only 1·4% more positive than the \( ^{13}\text{C} \) value of starch isolated from the leaf. The respiratory contribution to discrimination has been extensively discussed by Francey et al. (1985), who also concluded that this term was probably small.

Another source of uncertainty is the effective value of \( b \). Farquhar and Richards (1984) suggested that allowance could be made for fractionation of \( \text{CO}_2 \) by PEP carboxylations which occur in \( C_3 \) species. Taking \( b \) as the net fractionation caused by carboxylations, they wrote

\[
b = (1 - \beta)b_3 + \beta b_4 = b_3 - \beta(b_3 - b_4),
\]

where \( \beta \) is the molar proportion of carbon fixed by PEP carboxylation. With the most recent estimate of \( b_3 \) being 29% at physiological pH and 25°C (Roeske and O’Leary 1984), and keeping \( b_4 \) as \( -5\cdot7\% \), then a \( \beta \) of 0·05 (Holbrook et al. 1984) corresponds to \( b = 27\cdot3\% \). The calculations here have been made with \( b = 27\% \). However, variation in \( \beta \) would affect the result to some extent.

There is also uncertainty associated with the drop in \( p(\text{CO}_2) \) between the intercellular spaces and the sites of carboxylation, due to finite transfer resistance, \( r \). From equation (7),

\[
r = (\text{slope in Fig. 3})/(b - b_1 - a_1) = 61/25\cdot2 = 2\cdot4 \text{ m}^2 \text{ s bar mol}^{-1}
\]

for \( T. \) aestivum. An estimate for the \( \text{CO}_2 \) transfer resistance in \( T. \) aestivum has previously been obtained from the relationship between the slope of the function relating \( \text{CO}_2 \) assimilation rate to intercellular \( p(\text{CO}_2) \) (at limiting \( p(\text{CO}_2) \) and high irradiance) and the \textit{in vitro} RuP2 carboxylase activity. Analysis of the curvature in that relationship yielded a value of 2·0 ± 0·1 m\(^2\) s bar mol\(^{-1}\) for the \( \text{CO}_2 \) transfer resistance (Evans 1983a).

There is some uncertainty in the value as the method involved the assumption that the \( \text{CO}_2 \) transfer resistance was independent of leaf nitrogen content and growing season. Nevertheless, the two independent estimates for the transfer resistance in \( T. \) aestivum are in good absolute agreement. Subsequent work with \textit{Hordeum} using discrimination measured over the short term has yielded a similar range of values (2·3 ± 1·9 m\(^2\) s bar mol\(^{-1}\)) for \( r \); however, a plot of the \textit{Hordeum} data as in Fig. 3 yielded a negative slope (K. Hubick and G. Farquhar, unpublished data). From Fig. 3, the value for \( T. \) monococcum could be as low as 1·2 m\(^2\) s bar mol\(^{-1}\).

Various theoretical calculations of the resistance to \( \text{CO}_2 \) diffusion between the intercellular airspaces and the sites of carboxylation have been made. The two dominant factors which emerge are the permeability of the plasmalemma to \( \text{CO}_2 \) and the surface area of chloroplasts adjacent to intercellular air spaces per unit leaf area. Estimates of resistance on a cell wall basis range from 1·4 s cm\(^{-1}\) (Čásky and Ticha 1982), 6 s cm\(^{-1}\) (Nobel 1974) to 10·15 s cm\(^{-1}\) (Raven and Glidewell 1981) which correspond to 0·2-0·7, 1 and 1·7-2·5 m\(^2\) s bar mol\(^{-1}\), respectively, if a value of 15 is taken for the exposed surface area ratio and standard atmospheric pressure is used. The latter two and those discussed above all point to a resistance between 1 and 2·5 m\(^2\) s bar mol\(^{-1}\) The consequence of the \( \text{CO}_2 \) transfer resistance is that, for a wheat leaf with a rate of \( \text{CO}_2 \) assimilation of 30 \( \mu \text{mol m}^{-2} \text{ s}^{-1} \), the \( p(\text{CO}_2) \) at the sites of carboxylation is about 60 \( \mu \text{bar} \) below \( p_i \), so that \( p_c/p_a = 0·52 \) and \( p_i/p_a = 0·7 \). This drawdown needs to be taken into account when calculating the performance of RuP2 carboxylase \textit{in vivo}. 
The observed discrimination against $^{13}\text{C}_2$ by the flag leaf of *T. aestivum* with a $p_i/p_a$ of 0.7 was about 16% (Fig. 1). This corresponds to the $\Delta$ value of wheat grains (15.4–16.4%, Farquhar and Richards 1984) which would be produced by carbon exported by the flag leaf and differs from the $\Delta$ value of the leaves (18.9–20.5%). In grape leaves the isotopic composition, $\delta$, of aqueous extract was 2% more positive than that of whole leaves (Di Marco *et al.* 1977), i.e. currently fixed carbon effectively shows less discrimination than is seen in the structural carbon. This difference could be due to higher $p_i/p_a$ at times when structural carbon is laid down. Indeed high $p_i/p_a$ is sometimes reported in juvenile leaves (Čatský *et al.* 1976; Woodward and Rawson 1976; Rawson and Constable 1981. Evans (1983b) excluded light from young growing wheat leaves and showed that $\delta$ from this material was 1% more positive than that from leaves grown in the light. A simple explanation is that leaves forming in the light supplement the import of carbon with carbon fixed by the developing leaves at a larger $p_i/p_a$ than is found in mature leaves. A similar argument has been made for pine trees, where the $\delta^{13}\text{C}$ value of wood is usually 2% less negative than that of the needles. Rather than invoking further discrimination during the synthesis of lignin, the $\delta^{13}\text{C}$ value of wood may reflect carbon export from mature needles with lower $p_i/p_a$ (Evans 1983b; Francey *et al.* 1985).

For the C₄ species in Fig. 2, the discrimination appears nearly independent of $p_i/p_a$. Present theory suggests that a portion, $\phi$, of the CO₂ which reaches the bundle sheath chloroplasts subsequently escapes fixation, allowing some fractionation by RuP₂ carboxylation to match that, of an opposite sign, due to bicarbonate equilibration with CO₂ and PEP carboxylation, and leaving only the fractionation due to diffusion obvious. The leakage, $\phi$, may be estimated from the data according to equation (2) as 0.34 and 0.21 for *A. edulis* (NAD–ME dicot) and *Z. mays* (NADP–ME monocot), respectively. Since the ratio, $\xi$, (cf. equation (A7)) was between 3 and 5 and the precision of the $\delta^{13}\text{C}$ values was about 0.1%, difference between the C₄ types is barely resolvable. However, the present values are in agreement with data for whole leaves which predict leakages of 0.37 and 0.27 for the two species, respectively (Hattersley 1982).

It is obvious from this discussion that many aspects of carbon isotope fractionation are, at this stage, of uncertain magnitude. Nevertheless, the present technique for ‘online’ measurement of discrimination has confirmed one aspect of fractionation theory — the importance of diffusional and carboxylation components. The new technique is likely to be refined. If so, it will add to the uses of carbon isotope ratio mass spectrometry. These already include identification of photosynthetic pathway, estimation of average intercellular CO₂ concentration, and identification of differences in water-use efficiency. To these we tentatively add the estimation of transfer resistances within the leaf.

**Acknowledgments**

Thanks are due to Sue Wood and Zarko Roksandic for preparing the $\delta^{13}\text{C}$ leaf samples and ratio mass spectrometry, to Yvonne Williams and Jane Vickers for the difficult typing, and to Tony Condon and Kerry Hubick for helpful comments.

**References**


Appendix

Carbon Isotope Discrimination

in a Gas-exchange Cuvette

The rate of entry of \( ^{12}CO_2 \) into a well mixed gas-exchange cuvette is \( uc_e \), where \( u \) is the rate of flow of air (mol s\(^{-1}\)) and \( c_e \) is the concentration (mole fraction) of \( ^{12}CO_2 \) in air. This rate equals that at which \( ^{12}CO_2 \) leaves the cuvette at concentration \( c_o \), together with the rate of assimilation of \( ^{12}CO_2 \) by the leaf, \( sA \), where \( s \) (m\(^2\)) is the projected leaf area and \( A \) (mol m\(^{-2}\) s\(^{-1}\)) is the rate per unit area. Thus,

\[
uc_e = uc_o + sA.
\]  

(A1)

It is important to note that the flow rates and concentrations of CO2 are here expressed as the values at a standard humidity (typically either zero humidity, or in equilibrium with an ice-water mixture), since otherwise the increased flow rate of wet air, and dilution of CO2, by transpiration will complicate the expression.

Conservation of \( ^{13}CO_2 \) is expressed as

\[
uR_e c_e = uR_o c_o + s(A^{13}/A)A,
\]  

(A2)

where \( R_e \) is the \( ^{13}C/^{12}C \) ratio of air entering the chamber, \( R_o \) that of air leaving the well-mixed chamber, and \( A^{13} \) is the rate per unit area of assimilation of \( ^{13}CO_2 \).

Following Farquhar and Richards (1984), we define discrimination in a process as the isotopic ratio of the source divided by that of the product, so that ‘discrimination against \( ^{13}CO_2 \) in air moving past a leaf’, \( \Delta_a \), is

\[
\Delta_a = R_e/R_o - 1,
\]  

(A3)

and discrimination by the leaf against uptake of \( ^{13}CO_2 \) is

\[
\Delta = \frac{R_o}{A^{13}/A} - 1.
\]  

(A4)

To obtain an expression for \( R_e/R_o \) (and hence \( \Delta_a \) from equation (A3)), we divide (A2) by \( uc_e R_o \) to obtain

\[
\frac{R_e}{R_o} = \frac{c_o}{c_e} + \frac{s(A^{13}/A)A}{uc_e R_o}.
\]  

(A5)

The factor \( (A^{13}/A)/R_o \) may be replaced, using equation (A4), by \( 1/(1 + \Delta) \) so that equation (A5) becomes

\[
\frac{R_e}{R_o} = \frac{c_o}{c_e} + \frac{1}{(1 + \Delta)\xi},
\]  

(A6)

where

\[
\xi = uc_e/(sA)
\]  

(A7)
and $\xi$ is the ratio of rate of CO$_2$ entry into the chamber to that of CO$_2$ entry into the leaf (assimilation). An equivalent expression for $\xi$ is found by dividing equation (A1) by $uc_e$ (again remembering that $c_e$ and $c_0$ are measured at a standard humidity):

$$1 = \frac{c_0}{c_e} + \frac{1}{\xi} \quad \text{(A8)}$$

i.e.

$$\xi = \frac{c_e}{(c_e-c_0)} \quad \text{(A9)}$$

The term $c_0/c_e$ in equation (A6) may be replaced using equation (A8) yielding

$$\frac{R_e}{R_0} = 1 - \frac{1}{\xi} + \frac{1}{(1+\Delta)\xi} = 1 - \frac{\Delta}{\xi(1+\Delta)}$$

Thus, from equation (A3),

$$\Delta_a = -\frac{\Delta}{\xi(1+\Delta)} \quad \text{(A10)}$$

Note that $\Delta$ is typically less than $30 \times 10^{-3}$, i.e. much less than 1, so that discrimination seen by the air is approximately $\xi$ times less than that of the leaf, and of opposite sign. If $\Delta_a$ is measured then $\Delta$, the leaf property, is determined by rearranging equation (A10) as

$$\Delta = -\frac{\xi \Delta_a}{1 + \xi \Delta_a} \quad \text{(A11)}$$

Note, again, that $\xi \Delta_a$ is much less than 1, so that $\Delta$ is approximately $\xi$ times $\Delta_a$, and of opposite sign.

In principle, $\Delta_a$ can be measured directly on an isotope-ratio mass spectrometer, comparing the isotopic ratios of CO$_2$ trapped from air before and after the cuvette. Sometimes it is convenient to measure them separately and compare each to some standard, of isotopic ratio $R_s$, say. Using standard $\delta$ notation ($\%$), this means

$$\delta_e /1000 = \frac{R_e}{R_s} - 1 \quad \text{(A12)}$$

and

$$\delta_0 /1000 = \frac{R_0}{R_s} - 1 \quad \text{(A13)}$$

and that

$$\Delta_a = \frac{\frac{R_e}{R_s}}{\frac{R_0}{R_s}} - 1 = \frac{\delta_e - \delta_0}{1000 + \delta_0}, \quad \text{(A14)}$$

so that equation (A11) becomes

$$\Delta = \frac{\xi (\delta_0 - \delta_e)}{1000 + \delta_0 - \xi(\delta_0 - \delta_e)} \quad \text{(A15)}$$

If the standard is PDB then $\delta_o$ is negative, but $\delta_e$ is even more negative.

Manuscript received 25 October 1985, accepted 9 December 1985