Internal Conductance to CO₂ Diffusion and C¹⁸OO Discrimination in C₃ Leaves

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¹⁸O discrimination in CO₂ stems from the oxygen exchange between ¹⁸O-enriched water and CO₂ in the chloroplast, a process catalyzed by carbonic anhydrase (CA). A proportion of this ¹⁸O-labeled CO₂ escapes back to the atmosphere, resulting in an effective discrimination against C¹⁸OO during photosynthesis (Δ¹⁸O). By constraining the δ¹⁸O of chloroplast water (δc) by analysis of transpired water and the extent of CO₂-H₂O isotopic equilibrium (θw) by measurements of CA activity (θw = 0.75–1.0 for tobacco, soybean, and oak), we could apply measured Δ¹⁸O in a leaf cuvette attached to a mass spectrometer to derive the CO₂ concentration at the physical limit of CA activity, i.e. the chloroplast surface (cₕ). From the CO₂ drawdown sequence between stomatal cavities from gas exchange (cₛ), from Δ¹⁸O (cₕ), and at Rubisco sites from Δ¹³C (cₐ), the internal CO₂ conductance (gₚ) was partitioned into cell wall (gₕ) and chloroplast (gₕw) components. The results indicated that gₕ is variable (0.42–1.13 mol m⁻² s⁻¹) and proportional to CA activity. We suggest that the influence of CA activity on the CO₂ assimilation rate should be important mainly in plants with low internal conductances.

Natural variation in ¹⁸O content (δ¹⁸O) of CO₂ is a useful tracer for photosynthetic activity. This is due to a sequence of events: first, δ¹⁸O of chloroplast water is high due to evaporative effects (Gonfiantini et al., 1965); second, in the chloroplasts, exchange of oxygen between CO₂ and H₂O is catalyzed by carbonic anhydrase (CA); and third, a large fraction of this ¹⁸O-labeled CO₂ diffuses from the chloroplast back to the atmosphere. On a leaf scale, this “retroflux” of ¹⁸O-enriched CO₂ from the leaf back to the atmosphere is observed as an enrichment in the C¹⁸OO in air passing over the leaf or as discrimination against C¹⁸OO by the leaf (Δ¹⁸O) (Farquhar and Lloyd, 1993). Notably, Δ¹⁸O is also observed on a global scale as latitudinal and seasonal changes in the δ¹⁸O of atmospheric CO₂. The quantitative use of such large-scale signals, however, still critically depends on better understanding of the basic processes influencing Δ¹⁸O (Francey and Tans, 1987; Farquhar et al., 1993; Ciais et al., 1997).

To interpret Δ¹⁸O measured during leaf-atmosphere CO₂ exchange, an estimate of CO₂ concentration at the site of CO₂-H₂O is required (Farquhar and Lloyd, 1993). The chloroplast CO₂ concentration (cₚ) may be derived from comparing measured and modeled discrimination against ¹³CO₂ (Δ¹³C) (Farquhar et al., 1982; Evans et al., 1986; von Caemmerer and Evans, 1991). Since both the photosynthetic enzyme Rubisco (responsible for ¹³C discrimination) and CA (responsible for Δ¹⁸O) are similarly distributed within the chloroplast stroma (Anderson et al., 1996), the ¹³C-derived value of cₚ was also applied to Δ¹⁸O (Farquhar et al., 1993; Flanagan et al., 1994). However, it was suggested (Yakir, 1998) that the CO₂ concentration pertaining to Δ¹⁸O may be associated with the chloroplast surface, i.e. the limit of CA activity, and not the mean CO₂ concentration at sites of CO₂ fixation by Rubisco. This is because CA acts to cancel out any gradients in ¹⁸O of CO₂ within its domain. We now suggest that with adequate estimates of chloroplast water δ¹⁸O and of the extent of CO₂-H₂O isotopic equilibrium in the chloroplast (i.e. CA activity), it should be possible to use Δ¹⁸O to accurately estimate the effective CO₂ concentration at the sites of CO₂-H₂O equilibrium. This approach is somewhat similar to that using observed and predicted Δ¹³C to compare cᵣ and cₚ (von Caemmerer and Evans, 1991).

Using ¹³C-derived estimates of cₚ, the internal leaf conductance to CO₂ (gₚ) and its influence on leaf photosynthesis have been well characterized (von Caemmerer and Evans, 1991; Lloyd et al., 1992; Loreto et al., 1992; Syvertsen et al., 1995). However, evaluating the relative importance of the major components of gₚ, the wall conductance (gₚw) and the chloroplast conductance (gₚch) has been restricted (Cowan, 1986; Evans et al., 1994). The association of Δ¹⁸O with CO₂ concentration at the chloroplast surface should enable this partitioning. Information on CA activity directly from assays or through Δ¹⁸O measurements should also provide insight into the role of CA in facilitating diffusion within the chloro-
plast (Cowan, 1986; Makino et al., 1992; Price et al., 1994; Williams et al., 1996).

By comparing the CO₂ concentration and isotopic composition of air entering and leaving a leaf chamber, dis- crimination against C¹³O₂ (Δ¹³O) may be measured "on-line" in a method equivalent to Δ¹³C (Evans et al., 1986):

\[ \Delta = \frac{\xi(\delta_0 - \delta_m)}{1,000 + \delta_0 - \xi(\delta_0 - \delta_m)} \cdot 1,000 \]  

(1)

where \( \xi = \frac{c_i}{(c_i - c_o)} \), \( c_i \) and \( c_o \) and \( \delta_i \) referring to the CO₂ concentration (corrected to the same humidity) and isotopic composition of air entering and leaving the cuvette, respectively. Δ¹³O can also be predicted (Farquhar and Lloyd, 1993) as

\[ \Delta_{18O} = \frac{\bar{a} + \epsilon\Delta_{18O}}{1 - \epsilon\Delta_{18O}/1,000} \]  

(2)

where \( \Delta_{18O} = 1,000\{(\delta_i / 1,000 + 1)/(\delta_i / 1,000 + 1) - 1\}; \epsilon = c_i/(c_i - c_o) \); \( \delta_o \) and \( \delta_c \) represent the δ¹³O of CO₂ in the overlying air and in full isotopic equilibrium with water in the chloroplast, respectively, and \( c_i \) and \( c_o \) the respective CO₂ concentrations (see Fig. 1); \( \bar{a} \) is the weighted-mean diffusional fractionation through the boundary layer, 5.8‰, stomata, 8.8‰, and aqueous leaf media, 0.8‰, (Farquhar and Lloyd, 1993). Despite general agreement between modeled and measured Δ¹³O (Farquhar et al., 1993; Flanagan et al., 1994; Williams et al., 1996), large quantitative discrepancies often occur (Yakir et al., 1994; Williams et al., 1996; Harwood et al., 1998; Wang et al., 1998). There are three main assumptions in Equation 2: (a) chloroplast water (and hence CO₂ in equilibrium with this water) is assumed to be isotopically similar to water at the evaporating sites (\( \delta_i \)); (b) CO₂ and H₂O in the chloroplast reach full isotopic equilibrium; and (c) \( c_i \) correctly represents the CO₂ concentration at the site of oxygen exchange.

The isotopic composition of water at evaporating surfaces (\( \delta_i \)) may be estimated from the Craig and Gordon model of evaporative enrichment (Craig and Gordon, 1965):

\[ \delta_i = \delta_s + e^* + e_k + h^* \cdot (\delta_s - e_k - \delta_i) \]  

(3)

where \( h^* \) is the relative humidity at leaf temperature; \( \delta_s \) and \( \delta_i \) are the isotopic composition of water vapor in the air and transpired by the leaf, respectively; \( e_k \) is the combined diffusional fractionation through stomata and turbulent boundary layer (Farquhar and Lloyd, 1993; Buhay et al., 1996); and \( e^* \) is the temperature-dependent liquid-vapor fractionation. The measurement of δ¹³O of transpired water vapor (\( \delta_i \)) allows estimation of \( \delta_i \) under non-steady-state conditions (Harwood et al., 1998). While the proximity of chloroplasts to the liquid-air interface in leaves implies good mixing between evaporating sites and chloroplasts, isotopic gradients in leaf water can occur.
of hydration reactions achieved per CO\(_2\) molecule (\(k\tau\)). This “coefficient of CO\(_2\) hydration” may be calculated for a leaf by calculating the rate constant (\(k\)) from biochemical measurements of CA activity and the residence time of CO\(_2\) in the leaf (\(\tau\)) from photosynthetic flux measurements of CO\(_2\) (see “Materials and Methods”). In this way, the extent of isotopic equilibrium may be directly determined.

We sought to determine the CO\(_2\) concentration relevant to CO\(_2\)-H\(_2\)O in leaves from measurements of \(\Delta^{18}O\) by constraining both the \(\delta^{18}O\) of exchangeable water and the extent of isotopic equilibrium as above. The subsequent implications toward internal CO\(_2\) conductance are discussed in the context of CA activity and its role in facilitating diffusion of CO\(_2\) within the chloroplast.

RESULTS AND DISCUSSION

Interpreting C\(^{18}\)OO discrimination requires information on \(\delta^{18}O\) of water in the chloroplasts, the extent of isotopic equilibrium between CO\(_2\)-H\(_2\)O and the CO\(_2\) concentration in the chloroplast. The \(\delta^{18}O\) value of chloroplast water is often derived from the Craig and Gordon model for evaporating water (\(\delta_e\) in Eq. 3; Flanagan et al., 1994; Williams et al., 1996; Yakir and Wang, 1996; Wang et al., 1998; Harwood et al., 1998). This is due to the proximity of chloroplasts to the liquid-air interfaces within leaves. This leaves two options in using Equation 2 and measurements of \(\Delta^{18}O\): (a) to use \(\Delta^{13}C\)-derived estimates of \(c_c\) as the CO\(_2\) concentration relevant to the site of oxygen exchange in the chloroplast and solve for the extent of isotopic equilibrium (e.g., Flanagan et al., 1994); and (b) to independently measure the extent of isotopic equilibrium and solve for \(c_c\). We argue that \(c_c\) does not refer to the site of CO\(_2\)-H\(_2\)O equilibrium and so took the latter approach to estimate its true value. Using constrained estimates of \(\delta_e\) and direct assays of CA activity, we solved both \(\Delta^{18}O\) and \(\Delta^{13}C\) discrimination equations for \(c_c\). We found the \(\Delta^{18}O\)-derived \(c_c\) to be always higher than \(\Delta^{13}C\)-derived values, and define the CO\(_2\) concentration relevant to \(\Delta^{18}O\) as \(c_{cs}\) (for [CO\(_2\)] at the chloroplast surface). We then use \(c_{cs}\) to partition the internal conductance into its two major components. In the following sections we show how the interpretations were constrained and discuss their implications.

Observed \(\Delta^{18}O\)

Consistent with previous observations and predictions, a clear dependence of \(\Delta^{18}O\) on \(c_c/c_a\) was observed (Fig. 2; Farquhar et al., 1993; Flanagan et al., 1994; Williams and Flanagan, 1996; Williams et al., 1996). As expected, \(\Delta^{18}O\) was also larger when measured using \(^{18}\)O-depleted source CO\(_2\), which generated a larger isotopic difference between source and leaf CO\(_2\) (\(\Delta_{ca}\)) (increasing the precision of measurement). No difference in the response was observed under different photosynthetic conditions in oak leaves (Fig. 2c). Low pO\(_2\) did, however, induce higher assimilation rates and lower \(c_i\) and \(c_c\) (Table I) due to reduced photosynthesis. Estimates of internal CO\(_2\) conductance (\(g_i\)) derived from \(\Delta^{13}C\) measurements were 0.50 (±0.12), 0.32 (±0.05), and 0.27 (±0.09) mol m\(^{-2}\) s\(^{-1}\) for tobacco, soy, and oak respectively, which were used to calculate \(c_c/c_a\). Our \(\delta_e\) estimates were in line with previous estimates on similar herbaceous
Table I. Gas exchange (at max PPFD), CA activity, and isotopic water data (average at all PPFDs) for tobacco, soy (n = 3 and 2), and oak (n = 3, at different O₂%)

Units: evaporation rate (Eₘₐₓ, mmol m⁻² s⁻¹), stomatal CO₂ conductance (gₘₐₓ, mmol CO₂ m⁻² s⁻¹), CO₂ assimilation (Aₘₐₓ, μmol m⁻² s⁻¹), sub-stomatal CO₂ concentration (cₑ, μmol mol⁻¹), leaf temperature (Tₑₘₐₓ °C), carbonic anhydrase CO₂ hydration rate, under assay (CAₐₙₙ, μmol CO₂ m⁻² s⁻¹), at 2°C and 35 mM [CO₂], mean of three leaves), and in vivo conditions (CAₑₙₑ, at cₑ and Tₑₙₑ, μmol CO₂ m⁻² s⁻¹). For VPD (kPa) and isotopic data (%), nos. are averages (+sd) during the entire light response. Symbols as in text.

<table>
<thead>
<tr>
<th>Species</th>
<th>Eₘₐₓ</th>
<th>gₘₐₓ</th>
<th>Aₘₐₓ</th>
<th>cₑ(min)</th>
<th>Tₑₘₐₓ</th>
<th>CAₐₙₙ</th>
<th>CAₑₙₑ</th>
<th>VPD</th>
<th>δₑ</th>
<th>δₛ</th>
<th>δₑₑ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco 1</td>
<td>5.5</td>
<td>244</td>
<td>14.1</td>
<td>274</td>
<td>27.2</td>
<td>n.d.</td>
<td>1,623</td>
<td>1.37 (0.19)</td>
<td>-6.3(0.9)</td>
<td>+12.0(1.1)</td>
<td>+11.0</td>
</tr>
<tr>
<td>Tobacco 2</td>
<td>4.8</td>
<td>189</td>
<td>13.5</td>
<td>293</td>
<td>54.8(13)</td>
<td>598</td>
<td>1.48 (0.17)</td>
<td>-5.5(0.6)</td>
<td>+15.6(1.0)</td>
<td>+11.9</td>
<td></td>
</tr>
<tr>
<td>Tobacco 3</td>
<td>5.0</td>
<td>241</td>
<td>15.8</td>
<td>265</td>
<td>28.5</td>
<td>71.3(6.3)</td>
<td>670</td>
<td>1.27 (0.18)</td>
<td>-2.3(0.7)</td>
<td>+16.4(1.1)</td>
<td>+13.6</td>
</tr>
<tr>
<td>Soy 1</td>
<td>4.3</td>
<td>206</td>
<td>12.2</td>
<td>255</td>
<td>29.3</td>
<td>25.3(2.9)</td>
<td>294</td>
<td>1.51 (0.11)</td>
<td>-4.7(0.9)</td>
<td>+11.0(1.1)</td>
<td>+11.0</td>
</tr>
<tr>
<td>Soy 2</td>
<td>5.1</td>
<td>300</td>
<td>20.0</td>
<td>237</td>
<td>28.5</td>
<td>37.1(1.6)</td>
<td>342</td>
<td>1.32 (0.14)</td>
<td>-5.1(0.9)</td>
<td>+12.7(0.3)</td>
<td>+11.9</td>
</tr>
<tr>
<td>Oak - 40%</td>
<td>3.3</td>
<td>142</td>
<td>11.8</td>
<td>237</td>
<td>28.9</td>
<td>n.d.</td>
<td>-1</td>
<td>1.62 (0.18)</td>
<td>-5.6(1.9)</td>
<td>+13.4(1.4)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Oak - 20%</td>
<td>4.2</td>
<td>166</td>
<td>13.5</td>
<td>207</td>
<td>29.2</td>
<td>n.d.</td>
<td>-1</td>
<td>1.57 (0.17)</td>
<td>-6.6(0.6)</td>
<td>+11.8(0.8)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Oak - 2%</td>
<td>4.1</td>
<td>172</td>
<td>17.5</td>
<td>174</td>
<td>27.8</td>
<td>n.d.</td>
<td>-1</td>
<td>14.6 (2.2)</td>
<td>-6.2(0.7)</td>
<td>+13.1(1.3)</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

*In vivo CA hydration based on the mean CAₐₙₙ of the other two tobacco plants.

δ¹⁸O of Water at the Site of Oxygen Exchange

Estimates of evaporating surface water (δₑₑ) were based on direct measurements of transpired water vapor (δₑₑ) applying the isotopic fractionation during evaporation (Eq. 3). The maintenance of constant vapor pressure deficit (VPD) during the complete photosynthetic photon flux density (PPFD) curve kept both δₑₑ and δₛ constant throughout each experiment (Table I). Determining δₑₑ in this way from δₑₑ avoids uncertainties that arise in substituting source water δ¹⁸O for δₑₑ in Equation 3, as shown in Table I; although δₑₑ is at steady state, the absolute value may differ from source water (−4.5‰) by several per mill. δₑₑ is assumed to provide a close approximation to δ¹⁸O of water in the chloroplasts. The proximity of chloroplasts to evaporating surfaces is sufficient to ensure good isotopic mixing between the two. In particular, this assumption would be safe when ¹⁸O heterogeneity in the entire leaf water is small. As a precautionary measure, such heterogeneity was evaluated by comparing δₑₑ with measured bulk leaf water (δₑₑₑₑ) both at the end of each light response experiment (Table I; Fig. 3, white symbols) and across a range of evaporation rates in an independent test (Fig. 3, black symbols). On average, bulk leaf water was lower than δₑₑ by 2‰ (±1‰ in Fig. 3). This phenomenon has been observed extensively (Wang et al., 1998, and refs. therein) and has been partly explained by the inclusion of unenriched vein water, estimated to represent 2% to 5% of total leaf water (Yakir et al., 1989; Flanagan et al., 1991), and/or by a Peclet effect proposed by Farquhar and Lloyd (1993).

The difference between δₑₑ and δₑₑₑₑ increased with the evaporation rate (Fig. 3, excluding the three marked data points, from leaves thought not to be at isotopic steady state), which is consistent with a Peclet effect (Flanagan et al., 1991; Farquhar and Lloyd, 1993). In this case, the large advective flux of water through the leaf at higher evaporation rates restricts back-mixing of ¹⁸O-enriched water from the evaporation sites with the bulk leaf water. The maximal Peclet effect observed here was 3‰ (excluding marked points), which was much smaller than those reported previously (Flanagan et al., 1991, 1994; Wang et al., 1998). The small Peclet effect in this
study was probably due to low evaporation rates (<5 mmol H₂O m⁻² s⁻¹, Table I). In addition, although not measured here, Peclet effects in oak (K.G. Harwood, D. Yakir, J.S. Gillon, and H. Griffiths, unpublished data) and other woody species (birch and poplar; Roden and Ehleringer, 1999) have been consistently equally low in a wide range of conditions. Therefore, the absence of significant isotopic gradients in leaf water over the whole leaf is a good indication that isotopic gradients are unlikely to occur across the much smaller distances (<0.01 mm) between evaporating surfaces and chloroplasts.

**Extent of CO₂-H₂O Isotopic Equilibrium**

We measured CA activity in the experimental plants and used the results to estimate the in vivo extent of isotopic equilibrium between CO₂ and H₂O in the chloroplast. Previously, close to full isotopic equilibrium has been assumed due to the high rates of CA catalysis expected in most plants. We tested this assumption by measuring CA activities under assay conditions and estimating in vivo rates under this assumption by measuring CA activities under equilibrium has been assumed due to the high rates in the chloroplast. Previously, close to full isotopic equilibrium (δe) from Equation 4, solid line was calculated from the CA activity and CO₂ residence time (kt), which represents the number of hydrations per CO₂ molecule and is related to Δc/Δe.

![Equation 4](image)

\[ \theta_{eq} = \frac{\Delta c}{\Delta e} \]

**Figure 4.** Diagram showing the dynamics of oxygen isotope exchange between atmospheric CO₂ (δa) and leaf water (δl) and the resulting δ¹⁸O of CO₂ (δc). Isotopic equilibrium (θeq) from Equation 4, solid line was calculated from the CA activity and CO₂ residence time (kt), which represents the number of hydrations per CO₂ molecule and is related to Δc/Δe.

CO₂ assimilation (μmol m⁻² s⁻¹)

![Extent of isotopic equilibrium, θeq](image)

**Figure 5.** The number of hydration reactions per CO₂ molecule (kt) calculated from \( \frac{CA_{leaf}}{F_{m}^{eq}} \) as a function of the CO₂ assimilation rate. Shown on the second axis is the equivalent extent of isotopic equilibrium (θeq) from Equation 4, in which full equilibrium (θeq ≈ 1) occurs above kr = 15. White symbols, Soy; black symbols, tobacco (different symbols refer to different light responses). All values for oak were above kr = 15 because of the assumed high CA rates.
gested that the equilibrium was 75% complete. In the extreme case, potentially high CA activity in oak corresponded to complete isotopic equilibrium throughout the light response, where $k_T$ was always greater than 15 (data not plotted in Fig. 5). Note that in other plants, including C3 and C4 species, $\theta_{eq}$ values were found to span the whole range from 0 to 1 (J.S. Gillon and D. Yakir, unpublished data).

**CO$_2$ Concentration at the Site of Isotopic Equilibrium**

Based on the above discussion that $\delta_a$ and $\delta_e$ and reflects $\theta_{eq}$, it is possible to show that $\theta_{eq}$ is related to $\Delta_{ca}/\Delta_{ea}$ (Fig. 4), since algebraically:

$$\theta_{eq} = \frac{R_c - R_e}{R_e - R_a} = \frac{1 - \frac{\alpha}{\epsilon + 1}}{\frac{1}{\epsilon + 1}} = \frac{\Delta_{ca} + \frac{\alpha}{\epsilon + 1}}{\Delta_{ea} + \frac{\alpha}{\epsilon + 1}}$$  \hspace{1cm} (5)

This describes the $^{18}$O/$^{16}$O ratio of CO$_2$ at the site of oxygen exchange ($R_e$) relative to that in full equilibrium with leaf water ($R_e$), and that for un-equilibrated CO$_2$ inside the leaf, $R_a$. The term $R_e' = R_e \cdot (1 - \frac{\alpha}{\epsilon + 1})$ allows for the variable expression of $\alpha$ under non-equilibrium conditions (as in $^{13}$C discrimination). Thus, $\left(1 - e^{kT/2}\right) = \theta_{eq} = \frac{\Delta_{ca}}{\Delta_{ea}}$ (Fig 5). We now incorporate the extent of isotopic equilibrium into Eq. 2, and C$^{18}$OO discrimination is then given as:

$$\Delta^{18}O = 1,000 \frac{\alpha + \epsilon}{1,000 - \epsilon} \left[\theta_{eq}\Delta_{ea} - (1 - \theta_{eq})\frac{\alpha}{\epsilon + 1}\right]$$  \hspace{1cm} (6)

With measured values of $\Delta^{18}O$, $\Delta_{ea}$, and $\theta_{eq}$, we may thus derive $\epsilon$, and hence $c_{c(eff)}$, the effective CO$_2$ concentration at the site of CO$_2$-H$_2$O equilibrium.

**Figure 6.** Data shown for all light response curves, showing internal CO$_2$ concentration ($\mu$mol mol$^{-1}$) as a function of CO$_2$ assimilation rate ($A$) ($\mu$mol m$^{-2}$ s$^{-1}$). Symbols refer to CO$_2$ concentration in internal air space (ci) (from gas exchange measurements, $\Delta_e$), $c_{c(eff)}$ (from $\Delta^{18}$O, $\delta_e$, and CA activity; *), and $c_c$ (from $\Delta^{13}$C; ♦). Species are tobacco (a–c), soy (d–e), and oak (f–h). Light responses in c and e were conducted using ambient $\delta^{18}$O CO$_2$, whereas the rest used CO$_2$ depleted in $^{18}$O. f through h, Experiments in 40%, 20%, and 2% $O_2$, respectively.
The $c_{c(eff)}$ values obtained from Equation 6 were always intermediate between $c_i$ (from gas exchange) and $c_c$ (from $\Delta^{13}$C), as shown in Figure 6. These results were obtained from eight experiments in three species, measured on two separate systems, reducing the likelihood of bias introduced via system or species effects. Typically, all values of internal [CO$_2$] dropped at high assimilation rates, as CO$_2$ demand increased, albeit with variation due to some non-correlated changes in stomatal conductance, particularly for soy. Species differences were evident: $c_{c(eff)}$ was generally closer to $c_i$ in soy, closer to $c_c$ in oak, and intermediate in tobacco.

The values of $c_{c(eff)}$ represent the CO$_2$ concentration at the effective site of CO$_2$-H$_2$O equilibrium, which we term $c_{cs}$ (Yakir, 1998), indicating that the chloroplast surface is the likely site. This assumes that the limit of CA activity occurs at the chloroplast surface, since the majority of CA resides within the chloroplast (Everson, 1970). These results are consistent with the difference between the effects of Rubisco on $\Delta^{13}$C and CA on $\Delta^{18}$O: although Rubisco and CA show the same distribution within the chloroplast (Anderson et al., 1996), Rubisco removes $^{12}$CO$_2$ from the system, creating a $^{13}$C gradient between $c_c$ and $c_c$. CA only acts to cancel out any $^{18}$O gradients in CO$_2$ throughout the domain of its activity, so that an $^{18}$O gradient only exists from the chloroplast surface ($c_{cs}$) to the atmosphere ($c_i$) (Fig. 7).

Note that in interpreting $\Delta^{18}$O, the best-constrained value is $\delta_c$. Consequently, testing the model for $\Delta^{18}$O usually involved deriving $\delta_c$ values and comparing them with $\delta_c$ values. Assuming that we appropriately adjust $\delta_c$ for $\theta_{eq}$ and correctly estimate $c_{cs}$, the two values should match. In previous studies, $^{13}$C-derived values of $c_c$ were used and the observed difference between $\delta_a$ and $\delta_c$ was explained in terms of incomplete isotopic equilibrium. The effects of incomplete equilibrium was addressed in those cases by applying a certain $\rho$ value ($\rho = A/CA_{leaf}$), which was incorporated within the $\Delta^{18}$O model (Farquhar and Lloyd, 1993; Flanagan et al., 1994; Williams and Flanagan, 1996; Williams et al., 1996). Furthermore, the method of $c_{c}$ determination based on individual $\Delta^{13}$C measurements (and not the trend of $\Delta^{13}$C across the full range of $A$) generated a range of $c_c$ values (up to 40 mmol mol$^{-1}$), so that co-adjustment of $c_c$ and $\rho$ was required to resolve $\delta_c$ versus $\delta_a$ differences.

In some cases, estimates of $\delta_a$ were as much as 10‰ below $\delta_c$ in both laboratory and field studies (Yakir et al., 1994; Harwood et al., 1998; Wang et al., 1998). Such differences cannot be explained by only considering $c_{cs}$ and probably imply large heterogeneity in leaf water isotopic composition. Especially in the two latter field studies, Peclet effects may be much larger than observed in this study. It is possible that these discrepancies represent isotopic leaf water heterogeneity between water in the chloroplast and at the evaporating sites. Better characterisation of the oxygen exchange site may help future studies of significant leaf water gradients and Peclet effects.

**Partitioning Internal CO$_2$ Conductance**

The association of $\Delta^{18}$O with the [CO$_2$] at the chloroplast surface ($c_{cs}$) provides us with another reference point in the diffusion pathway from atmosphere to chloroplast in addition to probing $c_i$ via gas exchange (von Caemmerer and Farquhar, 1981) and $c_c$ from $\Delta^{13}$C analysis (von Caemmerer and Evans, 1991). From Fick’s law of diffusion, CO$_2$ concentration gradients are related to conductance by the general expression $A = g_x (c_i - c_s)$. Applying values of $c_{cs}$, we may partition the total conductance ($g_i$) into its components before and after the chloroplast surface by plotting $A$ versus ($c_i - c_{cs}$) and versus ($c_{cs} - c_c$). In each case, the inverse of the gradient refers to cell wall/plasmalemma conductance ($g_{w}$) and conductance within the chloroplast ($g_{ch}$), respectively (Fig. 7), assuming no significant resistance to CO$_2$ diffusion in the gaseous leaf interior (Evans et al., 1994). Despite a larger error in determining conductances from $\Delta^{18}$O compared with $\Delta^{13}$C, $g_{w}$ was significantly higher than $g_i$ for both tobacco and soy, and on the borderline of significance for oak (Table II). Comparing the values of $g_{ch}$ relative to $g_w$, the chloroplast conductance was estimated to be 0.8 (tobacco), 0.3 (soy), and 3.2 (oak) times the wall conductance ($g_{w}/g_{ch}$, Table II). The magnitude and species variability of $g_{ch}$ was much lower than previous theoretical estimations, where the wall conductance was thought to be the major limitation to diffusion, such that $g_{w}/g_{ch}$ was predicted to be from 4.8 (Evans et al., 1994) to 7.4 (Cowan, 1986). The occurrence of low chloroplast conductance was associated with low in vivo CA activities (soy), while potentially high CA activity in oak may be associated with high $g_{ch}$.
Importance of CA-Mediated Diffusion in \( g_{\text{ch}} \)

It is becoming increasingly evident that CA facilitates diffusion and therefore CO\(_2\) conductance within the chloroplast (Cowan, 1986; Makino et al., 1992; Price et al., 1994; Williams et al., 1996; Sasaki et al., 1998). This may be further supported by the association of CA activity with the relative magnitude of chloroplast conductance in the three species used here (Table II). However, in the past, modification of CA activity has revealed little or no change in photosynthetic rate (Price et al., 1994; Williams et al., 1996), indicating only a small effect on assimilation of CA mutant compared with wild-type plants (approximately 0.4 mol m\(^{-2}\) s\(^{-1}\)).

Applying the present ratio of \( g_{\text{ch}}/g_w \) (0.8) for wild-type tobacco plants, we may calculate the conductance for wild-type plants from their \( \Delta^{13}\text{C} \)-derived \( g_i \) values. Assuming this physical wall conductance is unchanged between wild-type and CA mutant plants (the antisense CA gene should have no other effects on leaf physiology and structure), we estimate a lower value of \( g_{\text{ch}}/g_w = 0.5 \) for the CA mutant tobacco plants, i.e. the reduction in \( g_i \) is due to reduction in \( g_{\text{ch}} \) only. Indicating the position

**Table II.** The breakdown of total leaf CO\(_2\) conductance \( g_{\text{leaf}} \) into its components, stomatal \( g_s \) (from gas exchange) and internal \( g_i \) (from \( \Delta^{13}\text{C} \), plus error from 95% confidence limits of the slopes)

With \( \Delta^{13}\text{O} \), \( g_i \) is further partitioned into wall conductance to \( C_{\text{cs}}, g_w \), and the residual conductance within the chloroplast, \( g_{\text{ch}} \). All units are mol CO\(_2\) m\(^{-2}\) s\(^{-1}\). The ratio of chloroplast to wall conductance is also shown \( g_{\text{ch}}/g_w \). Average CA\(_{\text{leaf}}\) activity is shown for comparison (\( \mu \)mol m\(^{-2}\) s\(^{-1}\)).

<table>
<thead>
<tr>
<th>Species</th>
<th>( g_{\text{ch}} )</th>
<th>( g_w )</th>
<th>( g_i )</th>
<th>( g_{\text{ch}}/g_w )</th>
<th>( g_{\text{ch}}/g_w )</th>
<th>CA(_{\text{leaf}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco</td>
<td>0.155</td>
<td>0.224</td>
<td>0.50</td>
<td>0.12 (0.33)</td>
<td>0.90</td>
<td>623</td>
</tr>
<tr>
<td>Soy</td>
<td>0.141</td>
<td>0.253</td>
<td>0.32</td>
<td>0.13 (0.45)</td>
<td>0.42</td>
<td>318</td>
</tr>
<tr>
<td>Oak</td>
<td>0.102</td>
<td>0.166</td>
<td>0.27</td>
<td>0.35 (0.04)</td>
<td>1.13</td>
<td>2,016</td>
</tr>
</tbody>
</table>

In the absence of direct CA measurements in the oak species used here, CA\(_{\text{leaf}}\) is estimated from the average CA activity observed other oak species (see text).

**Figure 8.** The potential change in CO\(_2\) assimilation rate (A) as a function of \( g_{\text{ch}} \) (oak and tobacco, solid lines). \( g_{\text{ch}} \) is normalized relative to a constant wall conductance \( g_w \) (0.35 and 1.2 mol m\(^{-2}\) s\(^{-1}\) for oak and tobacco, respectively). The changes in A are expressed relative to measured assimilation rates at actual conductance values, \( g_{\text{ch}}/g_w = 0.8 \) and 3.2 for tobacco \( (A = 12.7 \ \mu \text{mol m}^{-2} \text{ s}^{-1}) \) and oak \( (A = 13.7 \ \mu \text{mol m}^{-2} \text{ s}^{-1}) \), respectively. Also marked is the estimated \( g_{\text{ch}}/g_w \) (see text) of tobacco mutants lacking CA (Price et al., 1994; Williams et al., 1996), indicating only a small effect on assimilation relative to the wild-type tobacco.
of the CA-mutant plants on Figure 8, we predict only a 5% drop in CO₂ assimilation for the 90% to 95% reduction in CA activity, in agreement with reported results.

Two main points arise from this simple analysis. First, it appears that relative chloroplast conductance is proportional to CA activity across almost three orders of magnitude of CA activity, with a possible minimum at \( \frac{g_{ch}}{g_w} = 0.5 \), where all residual CO₂ diffusion will be un-facilitated (i.e. no CA effect). This strongly supports the occurrence of CA-mediated diffusion in the chloroplast. Second, although the oak plants used may not be completely representative of woody species, CA activity in woody plants in general may have been optimized over evolutionary time to compensate for low wall conductance (J.S. Gillon and D. Yakir, unpublished data). For example, in a preliminary survey, mean in vivo CA hydration rates were 1,090 and 390 μmol m⁻² s⁻¹ for trees/shrubs (n = 16) and herbaceous species (n = 12), respectively, which may correspond to a three-times increase in \( g_{ch} \) relative to \( g_w \). By extending such surveys to include conductance estimates (both internal and stomatal), or by manipulating CA activity in species with low internal conductance, the potential importance of CA in photosynthesis may prove to be substantially greater than currently assumed.

MATERIALS AND METHODS

Plant Material

Soy (Glycine max), tobacco (Nicotiana tabacum), maize (Zea mays), and sorghum (Sorghum bicolor) were grown from seed in a greenhouse under ambient light and temperature at Weizmann Institute of Science (WIS). The latter two species were only used in the test of leaf water heterogeneity to increase the scope of the test. Oak seedlings (Quercus robur) were provided by the Forestry Commission (UK) in 1991, and kept outside and well-watered in 1-L pots at Moorbank Botanical Gardens (University of Newcastle-upon-Tyne, UK), until required. Measurements were conducted on 6- to 10-week-old plants of soy and tobacco plants and on 5-year-old oak seedlings. Transfer of plant material was several days prior to the experiment to allow acclimatization to laboratory conditions.

System 1 (WIS): Gas Exchange

Figure 9 shows a scheme of the on-line isotope/gas exchange system at WIS. Synthetic air was mixed from N₂, CO₂, and O₂ cylinders using mass flow controllers (MKS Instruments, Andover, MA), and humidified by bubbling a variable portion of the airstream through water at room temperature (\( \delta^{18}O = -4.5\% \), therefore, vapor \( \cong -14.5\% \)), acidified with two drops of 80% (v/v) H₃PO₄. The airflow was split into reference and analysis airstreams, the latter flow range, 800 to 1,500 mL min⁻¹, was passed to a Parkinson “conifer pod” leaf cuvette (PLC) (model PLC3C, ADC Scientific, Hoddeson, UK), and flow was measured via another mass flow controller. Illumination was from a 250 W projector lamp (GEC, Cleveland), passing through a 3-cm depth of water to reduce infrared radiation. Incident radiation on the leaf was controlled by shading with a predetermined number of Miracloth filters (Calbiochem, San Diego). Absolute CO₂ and H₂O concentration in reference and analysis airstreams were monitored alternately via an infrared gas analyzer (model Li-6262, LI-COR, Lincoln, NE).
Isotopic Measurement of CO₂

The outflow of the leaf chamber after passing through the infrared gas analyzer (minimum 700 mL min⁻¹) was split, 100 mL min⁻¹ was pumped first through a dryer (Nafion, Perma Pure, Toms River, NJ), and then a sample loop (0.85 mL) was fitted onto a six-port, two-position valve (Valco Instruments, Houston). CO₂ was trapped at liquid N₂ temperatures for 30 s. After warming to room temperature, the sample was swept with helium carrier gas (120 mL min⁻¹; ultrapure, Gordon Gas and Chemicals, Tel Aviv) through a magnesium perchlorate drying trap and a 2-m packed column (sieve 5Å, 80/100 mesh, Alltech, Deerfield, IL) at 60°C. The large peaks of N₂ and O₂ that eluted first from the column were diluted via a gas diluter (Micromass, Manchester, UK), followed by the non-diluted sample CO₂. The gas was introduced into the source of a mass spectrometer (OPTIMA, Micromass) via an open split. ¹³C to ¹²C and ¹⁸O to ¹⁶O isotope ratios were measured from the integrated peak areas of masses 44, 45, and 46 normalized against a 30-s CO₂ reference pulse injected prior to each sample. Sample size was standardized by adjusting the cryogenic trapping time according to the CO₂ concentration in the outflow from the leaf chamber. N₂O was assumed to be constant in air (310 nmol mol⁻¹) and absent from “synthetic” air, so δ values were corrected accordingly (Freidli and Siegenthaler, 1988) and expressed in the small delta notation versus Vienna Pee Dee Belenite (VPDB) for ¹³C and VPDB-CO₂ for ¹⁸O. Precision for repeated sampling of CO₂ was 0.06‰ (δ¹³C) and 0.07‰ (δ¹⁸O).

Isotopic Measurement of Water Vapor

The remaining airflow from the leaf chamber was passed at positive pressure to a 0.61-cm o.d. stainless steel vacuum line (pressure <1 × 10⁻³ torr) in which CO₂ and water vapor were trapped from the airstream (3 min at 500 mL min⁻¹) in a coil cooled with liquid N₂. After trapping, the line was evacuated and the trap was heated with a flame, distilling both CO₂ and H₂O into a Pyrex side arm immersed in liquid N₂. After quantitative transfer, the Pyrex tube was flame sealed. The sample was left for CO₂ and H₂O into a Pyrex side arm immersed in liquid N₂. After quantitative transfer, the Pyrex tube was flame sealed. The sample was left for CO₂-H₂O determinations for 1 to 2 h before beginning measurements. During sampling, CO₂ and water vapor were cryogenically trapped for 15 min from an airstream of 200 mL min⁻¹, during which time photosynthetic parameters were averaged. This was repeated at various PPFDs (500–1000 μmol photons m⁻² s⁻¹, 10–12 steps) to cover the range of CO₂ assimilation from approximately 5 μmol m⁻² s⁻¹ to saturation, allowing the photosynthetic rate to stabilize between each change in PPFD (approximately 20 min). The leaf-to-air VPD was maintained as constant as possible (approximately 1.5 ± 0.2 kPa) throughout the experiment by drying a portion of the reference airstream with Drierite (W.A. Hammond, Xenia, OH). Reference CO₂ was collected between every three to four samples. The complete light response and isotopic analyses were conducted on the same leaf three times, each once at 2%, 21%, and 35% O₂ to check the influence of photorepiration rate (all other experiments at WIS were conducted at 21% O₂).

Experimental Procedure

A portion of leaf was placed in the chamber and illuminated for 1 to 2 h before beginning measurements. During sampling, CO₂ and water vapor were cryogenically trapped for 15 min from an airstream of 200 mL min⁻¹, during which time photosynthetic parameters were averaged. This was repeated at various PPFDs (500–1000 μmol photons m⁻² s⁻¹, 10–12 steps) to cover the range of CO₂ assimilation from approximately 5 μmol m⁻² s⁻¹ to saturation, allowing the photosynthetic rate to stabilize between each change in PPFD (approximately 20 min). The leaf-to-air VPD was maintained as constant as possible (approximately 1.5 ± 0.2 kPa) throughout the experiment by drying a portion of the reference airstream with Drierite (W.A. Hammond, Xenia, OH). Reference CO₂ was collected between every three to four samples. The complete light response and isotopic analyses were conducted on the same leaf three times, each once at 2%, 21%, and 35% O₂ to check the influence of photorepiration rate (all other experiments at WIS were conducted at 21% O₂).

Leaf Water Heterogeneity

A separate experiment was carried out at WIS to determine the suitability of the Craig and Gordon model to estimate the δ¹⁸O of bulk leaf water (δLw). A leaf was
placed in the cuvette and left for 1 h (the minimum time for the first measurement in the above light response experiments). A dry CO₂ sample from the leaf chamber was first collected in the stainless steel line by passing the airstream through an additional acetone/liquid N₂ trap at −70°C in the vacuum line. This was used to derive the δ¹⁸O CO₂ to be used for equilibration. Next, a water vapor sample from the leaf chamber was collected, as before, and the leaf portion in the cuvette was then excised immediately afterward, and placed in a vacutainer. Finally a reference water portion in the cuvette was then excised immediately after-use for equilibration. Next, a water vapor sample from the first measurement in the above light response experiment was collected in the stainless steel line by passing the airstream assuming Q₁₀ = 2 (Hatch and Burnell, 1990).

Coefficient of CO₂ Hydration (kᵣ)

Two main factors control the exchange of ¹⁸O between leaf water and CO₂. During photosynthesis, the gross CO₂ influx rate (Fₘ) regulates the residence time (τ) of CO₂ in the aqueous leaf medium, while the CA-catalyzed hydration of CO₂ (CAₗₙₜ) determines the efficiency of oxygen exchange during that time (Eq. 4). The rate constant for CA (k) is equivalent to CAₗₙₜ/εₗₙₜ and the CO₂ residence time (τ) is given by εₗₙₜ/Fₘ. Thus the product, kᵣ = CAₗₙₜ/Fₘ, relates to the number of hydration reactions per CO₂ molecule. Fₘ was determined from the product of external CO₂ concentration (cₑ) and total conductance to the site of CO₂-H₂O equilibrium (cₛgₛₑₜₐ₇ₑₙ), where gₗₙₜ is the combination of boundary (gₗₙₜ), stomatal (gₛₙₜ), and internal conductance to cₑₙₜₐ₇ₑₙ. Using the resistance analogy, gₗₙₜ = 1/(1/gₛₙₜ + 1.6/gₛₙₜ + 1.6/gₛₙₜ). Boundary values are quoted in the methods, stomatal values were taken from gas exchange measurements (converting from water to CO₂ via the factor of 1.6), and the internal conductance estimate is described below (and see “Discussion”). Note that Fₘ may be also defined as A(ε + 1) from rearranging where ε = cₛₑₙₜₐ₇ₑₙ/(cₛₑₙₜ + cₑₙₜₐ₇ₑₙ).

Photosynthetic Calculations

Photosynthetic parameters were calculated according to the method of von Caemmerer and Farquhar (1981). Due to the influence of oxygen on water vapor determination from infrared gas analyzers (Ludwig et al., 1998), measurements in 2% and 35% O₂ were corrected with an O₂-dependent calibration coefficient (determined from separate tests). Conductance was corrected for the ratio of stomatal density between upper and lower surfaces (K. Parkinson, CIRAS manual, PP Systems, Hitchin, UK), which were measured from epidermal impressions as 0.32, 0.21, and 0.0 for tobacco, soy, and oak, respectively. Boundary layer conductance to H₂O was measured for each species by dipping a leaf in a weak detergent solution, removing excess water, then measuring the evaporation rate in a darkened cuvette (1.1, 0.9 mol m⁻² s⁻¹ for tobacco and soy in a PLC conifer pod, and 3.0 mol m⁻² s⁻¹ for oak in PLC leaf chamber, respectively). Leaf temperature was allowed to vary with PPFD, so that maximal Tₗₙₜ at saturating PPFD was between 27°C and 29°C for all experiments (minimum Tₗₙₜ = 23°C). Tₗₙₜ was calculated from the energy balance, where radiation and transmission characteristics were either taken from the CIRAS manual for the system at UNUT or measured directly at WIS. The ratio of PPFD (Delta-Ohm, Padova, Italy) to total radiation (LL-COR) was determined for the light source, as well as the transmission of the cuvette windows. All of the above parameters can greatly influence the energy budget and hence the calculation of photosynthetic parameters, especially εᵣ whereas we stress that such rigorous determination of
all parameters was essential for interpreting plant isotope discriminations.

\[ \Delta^{13}C \text{ Estimation of } c_i \]

The additional reduction in CO₂ concentration from \( c_i \) to the chloroplast (\( c_a \)) was estimated from the difference between the simple model and the measured discrimination (\( \Delta_i - \Delta_{\text{obs}} \)) (Evans et al., 1986) as:

\[ \Delta_i - \Delta_{\text{obs}} = \left( \frac{b' - a}{} \right) \frac{A}{g_i} \]

(5)

where \( \Delta_{\text{obs}} \) is the discrimination measured in Equation 1, \( \Delta_i = a + (b' - a) \frac{c_i}{c_a} \) (Farquhar et al., 1982), where \( c_i \) and \( c_a \) refer to CO₂ concentration in the substomatal cavity and atmosphere, respectively, \( a \) is the fractionation during diffusion in air (4.4‰), and \( b' \) is the fractionation during carboxylations (29‰). \( A \) is the combined fractionation (+1.8‰) during dissolution (+1.1‰) and diffusion through the liquid phase (+0.7‰). Internal conductance was derived from the gradient of the \( \Delta_i - \Delta_{\text{obs}} \) response versus \( A/c_a \) measured concurrently with \( \Delta^{18}O \) during the light responses. This method avoids any influence from photorespiration (Gillon and Griffiths, 1997) and uncertainty in \( b' \). Measurements where \( A < 8 \mu \text{mol m}^{-2} \text{s}^{-1} \) were excluded to avoid the influence of dark respiration on \( \Delta^{13}C \) (Gillon and Griffiths, 1997).

Statistical Analysis

For the determination of total internal conductance (\( g_i \)) (from \( A/c_a \) versus \( \Delta_i - \Delta_{\text{obs}} \)) and wall conductance (\( g_w \)) (from \( A/c_a \) versus \( \Delta_{\text{obs}} - \Delta_{\text{obs}} \)), linear regressions were obtained by the least square method, also deriving 95% confidence limits for slopes, from which the error for each conductance estimate was derived (Sokal and Rohlf, 1981).

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LITERATURE CITED


