Effect of Changes in Leaf Water Oxygen Isotopic Composition on Discrimination Against $C^{18}O^{16}O$ During Photosynthetic Gas Exchange

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Abstract

Photosynthetic gas exchange measurements were combined with measurements of the carbon and oxygen stable isotopic composition of CO_2 after it passed over a leaf of *Phaseolus vulgaris* or *Senecio* spp. plants held in a controlled environment chamber. Calculations were then made of discrimination by the leaf against $^{13}CO_2$ and $C^{18}O^{16}O$. Leaves were maintained at different vapour pressure gradients in order to generate a range of leaf water $^{18}O/^{16}O$ ratios. The ^{18}O content of leaf water increased when plants were exposed to higher vapour pressure deficits. The observed $C^{18}O^{16}O$ discrimination values also increased with an increase in the leaf-air vapour pressure gradient and the associated change in leaf water $^{18}O/^{16}O$ values. In addition, the observed $C^{18}O^{16}O$ discrimination values were strongly correlated with values predicted by a mechanistic model of isotopic fractionation.

Introduction

During photosynthetic gas exchange, the $^{13}C/^{12}C$ ratio of atmospheric CO_2 is increased as air passes over a leaf (Evans *et al.* 1986). In C_3 plants, carbon isotopic discrimination occurs primarily because of differences in diffusion rates between $^{13}CO_2$ and $^{12}CO_2$, and because of differences in the rates that ribulose-1,5-bisphosphate carboxylase (Rubisco) uses the two molecules in the principal carboxylation reaction (Farquhar *et al.* 1989). The $^{18}O/^{16}O$ ratio of atmospheric CO_2 is also changed as air passes over a leaf. Exchange between oxygen in CO_2 and oxygen in chloroplast water has been suggested to be the primary process responsible for ^{18}O discrimination during photosynthesis (Francey and Tans 1987; Friedli *et al.* 1987).

Farquhar and Lloyd (1993, 1994) and Farquhar *et al.* (1993) described a mechanistic model of ^{18}O fractionation processes during CO_2 exchange. The model predicts that when the isotopic composition of chloroplast water is held constant, $C^{18}O^{16}O$ discrimination should vary with changes in the partial pressure of CO_2 in the chloroplast, which in turn is dependent on the ratio of photosynthetic capacity and stomatal and wall conductance. The stable isotopic composition of leaf and chloroplast water is not normally constant, however, but is altered by fractionation processes that occur during transpiration (for a review see Flanagan 1993). The $^{18}O/^{16}O$ ratio of leaf and chloroplast water depends primarily on the leaf-air vapour pressure gradient, the larger the gradient the higher the $^{18}O/^{16}O$ ratio of leaf water (Flanagan 1993). In the initial experimental tests of the mechanistic model of $C^{18}O^{16}O$ discrimination, comparisons were made between observed

and predicted $C^{18}O^{16}O$ discrimination when environmental conditions were altered to induce variation in the ratio of photosynthetic capacity and stomatal conductance and thereby cause large changes in the chloroplast CO_2 partial pressure (Farquhar *et al.* 1993). In the experiments described here, plant leaves were maintained at different leaf–air vapour pressure gradients in a controlled environment gas exchange chamber in order to generate a range of leaf water $^{18}O/^{16}O$ ratios. Measurements were then made of the carbon and oxygen isotopic composition of CO_2 before and after it passed over a leaf with a steady, leaf water oxygen isotopic composition. As expected, the observed $C^{18}O^{16}O$ discrimination values increased with an increase in the leaf–air vapour pressure gradients and the associated change in leaf water $^{18}O/^{16}O$ values. In addition, the observed $C^{18}O^{16}O$ discrimination values were strongly correlated with values predicted by the mechanistic model of isotopic fractionation.

Materials and Methods

Plant Material and Growing Conditions

Phaseolus vulgaris cv. UNS 117 plants were germinated in $0 \cdot 012 \ m^3$ pots that were made from polyvinylchloride pipe and contained a soil made from one part vermiculite to two parts Utah soil mix that has been previously described (Comstock and Ehleringer 1988). Plants were grown in a glasshouse at the University of Utah, Salt Lake City with supplemental lighting provided by an alternating bank of metal halide and sodium vapour lamps as previously described (Flanagan *et al.* 1991b).

Small rosettes of the closely related species *Senecio streptanthifolius, S. streptanthifolius* var. *rubricaulis* and *S. multilobatus* (Bain 1983; Barkley 1988; hereafter referred to as *Senecio* spp.) were collected at a variety of locations in Utah, the roots were washed and wrapped in moist paper and the plants shipped via air to Carleton University, Ottawa. Plants were put in $0 \cdot 1$ m diameter pots containing soil made from equal parts peat, vermiculite and perlite. Plants were grown in a glasshouse with supplemental lighting provided by a bank of fluorescent lights. Gas exchange experiments were started after the plants had been growing in the glasshouse for over two months. Only mature leaves, produced while the plants were in the glasshouse, were used in the experiments.

Gas Exchange Measurements, and CO₂ and Water Collection

In Salt Lake City measurements of CO_2 and water vapour flux were made using an open gas exchange system as previously described (Ehleringer 1983; Ehleringer *et al.* 1992). In Ottawa measurements of CO_2 and water vapour flux were made with an open gas exchange system similar to the one described in detail by Parkhurst and Mott (1990) (MPH 1000 Gas exchange system, Campbell Scientific Inc., Logan, Utah; ADC 225-MK 3 infrared gas analyser, Analytical Development Co., Hoddesdon, Herts, England). A mature leaflet of *P. vulgaris* or an entire leaf of *Senecio* spp. were sealed into the leaf chamber and maintained under conditions of controlled temperature, humidity, light and CO_2 concentration. In both gas exchange systems humidification of the leaf chamber was entirely the result of evaporation of water from the leaf, since only dry air entered the leaf chamber. Humidity levels were varied by altering the flow rate of dry air entering the leaf chamber. All gas exchange calculations were done using the equations of Ball (1987) and Caemmerer and Farquhar (1981). Boundary layer conductance in the leaf chamber was approximately $2 \ mol \ m^{-2} \ s^{-1}$.

The water vapour and CO_2 in the air of the leaf chamber was collected by passing a portion of the air exiting the chamber through ethanol-dry ice traps to collect the water vapour, and liquid nitrogen traps to collect the CO_2 (Evans *et al.* 1986; Ehleringer *et al.* 1992). Pressure in the trap line was maintained at approximately 5 kPa during CO_2 collection in order to avoid the condensation of oxygen. In experiments with *P. vulgaris*, the leaflet was removed from the chamber immediately after gas exchange measurements were complete, and placed in a glass tube which was sealed with a rubber stopper and wrapped with Parafilm. A stem or petiole sample was also collected at the same time and sealed in a glass tube. The plant samples were then frozen until water was extracted from the tissue using a cryogenic vacuum distillation apparatus.

Isotopic Analysis

Water samples were prepared for measurements of oxygen isotopic composition by converting the oxygen in water to CO$_2$ using the guanidine hydrochloride method (Wong *et al.* 1987). The CO$_2$ gas from the guanidine hydrochloride technique and the CO$_2$ samples collected from the gas trapping line were then analysed for carbon and oxygen isotopic composition on a Finnigan-MAT delta S gas isotope ratio mass spectrometer. Precision of the method for water samples was checked by making repeated measurements of SMOW. Precision was found to be $\pm 0 \cdot 12\%o$ during the course of this study. Precision of the measurements for CO$_2$ samples collected on the gas trapping line was checked by making repeated collection of air samples passed through the leaf chamber with no leaf present. In Salt Lake City the standard deviation for 14 measurements of $\delta^{13}C$ was $0 \cdot 17\%o$, in Ottawa the standard deviation for nine measurements of $\delta^{13}C$ was $0 \cdot 03\%o$.

Isotopic compositions were expressed using lower case delta notation:

$$\delta = \left[\frac{R_{\text{Sample}}}{R_{\text{Standard}}} - 1 \right], \tag{1}$$

where R is the molar ratio of the heavy to light isotope (e.g. $^{18}O/^{16}O$). The results are conveniently presented in parts per thousand ($\%o$). Stem water and atmospheric water vapour isotopic ratios were expressed relative to SMOW. The isotopic composition of CO$_2$ was expressed relative to that of the PDB standard.

Isotopic discrimination (Δ) during photosynthetic gas exchange was calculated from the isotopic composition of air leaving the leaf chamber with (δ_o) and without (δ_e) a leaf present, as shown below (Evans *et al.* 1986):

$$\Delta = \frac{\xi(\delta_o - \delta_e)}{1 + \delta_o - \xi(\delta_o - \delta_e)}, \tag{2}$$

where $\xi = c_e/(c_e - c_o)$ and c_e and c_o are the partial pressures of CO$_2$ in the air, when the air is dried, entering (e) and leaving (o) the chamber while a leaf was present. The value of ξ varied, over the range $2 \cdot 8 - 10 \cdot 6$, in experiments conducted at different humidity levels with *P. vulgaris*. The value of ξ varied, over the range $9 \cdot 3 - 12 \cdot 9$, in experiments conducted with *Senecio* spp. The isotopic measurements of CO$_2$ were corrected for the presence of N$_2$O using the method described by Caemmerer and Evans (1991). The CO$_2$ collections from air passing through the empty leaf chamber were made with a CO$_2$ partial pressure close to that of the air stream exiting the chamber when a leaf was present (approximately 35 Pa). This resulted in only small corrections for N$_2$O, typically $+0 \cdot 13\%o$ for $^{13}C/^{12}C$ and $+0 \cdot 14\%o$ for $^{18}O/^{16}O$.

Modelling Isotopic Discrimination

There are two major processes influencing discrimination against C^{18}O^{16}O during photosynthetic gas exchange (Farquhar and Lloyd 1993, 1994). The first process is fractionation that occurs during diffusion. The CO$_2$ molecules containing ^{18}O are heavier and, therefore, diffuse at a slower rate than C^{16}O$_2$. The second process is oxygen isotope exchange that occurs between CO$_2$ molecules and H$_2$O in the chloroplast. During the exchange reaction, the oxygen isotope ratio of CO$_2$ becomes enriched relative to that of chloroplast water. A portion of the CO$_2$ that enters the leaf and equilibrates with chloroplast water is not fixed and diffuses back out of the leaf with an altered oxygen isotope ratio. The amount of CO$_2$ that escapes from the leaf depends on the partial pressure of CO$_2$ in the chloroplast and resistances to diffusion within and outside the leaf. The oxygen isotope ratio of CO$_2$ leaving the leaf will depend on three factors:

(*1*) the oxygyen isotope composition of chloroplast water;

(*2*) leaf temperature, which influences the equilibrium fractionation factor for the CO$_2$-H$_2$O exchange reaction;

(*3*) fractionation during diffusion of CO$_2$ out of the leaf.

The model of Farquhar and Lloyd (1993, 1994) (equations described below) was used to predict the discrimination by leaves under controlled environmental conditions.

Discrimination during diffusion

The isotope effect (α) that occurs during diffusion can be defined as the ratio of the diffusion coefficients for $C^{16}O_2$ and $C^{18}O^{16}O$ molecules. For numerical convenience we present fractionation factors using discrimination notation (Δ), which is defined as the deviation of an isotope effect from unity as shown below (Farquhar *et al.* 1989):

$$\Delta = \alpha - 1.\tag{3}$$

Equation (4) describes the weighted average of the fractionation factors during the diffusion of $C^{18}O^{16}O$ from the atmosphere to the chloroplast:

$$\bar{a} = \frac{a_b(c_a - c_s) + a_s(c_s - c_i) + a_w(c_i - c_c)}{c_a - c_c},\tag{4}$$

where c is the partial pressure of CO_2, and the subscripts a, s, i and c refer to the atmosphere, leaf surface, intercellular air spaces and chloroplast, respectively. The symbol a represents the discrimination during diffusion of $C^{18}O^{16}O$ at various steps in the atmosphere-chloroplast pathway, where the subscript b refers to diffusion through the leaf boundary layer, s diffusion through the stomata, and w the combination of dissolution of CO_2 into water and diffusion of CO_2 through water. The values for the diffusional discrimination factors for $C^{18}O^{16}O$ are a_b (5·8‰), a_s (8·8‰) and a_w (0·8‰).

Isotope composition of leaf and chloroplast water

It is very difficult to measure the isotope composition of chloroplast water on a routine basis. For experiments in this paper we assume that chloroplast water has an isotopic composition identical to that of water at the evaporative sites within leaves. The isotope composition of water at the evaporative sites can be calculated using a model of isotopic fractionation, originally developed by Craig and Gordon (1965) for evaporation from the ocean. The model can be expressed in the following form (Flanagan *et al.* 1991*b*):

$$R_e = \alpha_* \left[\alpha_k \, R_s \left(\frac{e_i - e_s}{e_i} \right) + \alpha_{kb} \, R_s \left(\frac{e_s - e_a}{e_i} \right) + R_a \left(\frac{e_a}{e_i} \right) \right],\tag{5}$$

where R is the molar ratio of the heavy to light isotope and the subscripts e, s and a refer to water at the evaporation sites within leaves, stem water and atmospheric water vapour, respectively; e is the partial pressure of water vapour and the subscripts i, s and a refer to the leaf intercellular air spaces, the leaf surface and the ambient air, respectively; α_* is the equilibrium isotope effect. The regression equations listed by Majoube (1971) were used to calculate values for the parameter α_* at specific leaf temperatures. α_k is the kinetic isotope effect for diffusion through the stomatal pore which is determined by the relative rates of molecular diffusion of the light and heavy isotope molecules in air. The $^{16}O/^{18}O$ value for α_k is 1·0285 (Merlivat 1978). α_{kb} is the kinetic isotope effect in a boundary layer and the $^{16}O/^{18}O$ value for α_{kb} is 1·0189 (Farquhar *et al.* 1988).

The isotope ratio of water at the evaporative sites (R_e) was expressed relative to stem water, using upper case delta notation (Δ, discrimination):

$$\Delta = \left[\frac{R_{Sample}}{R_{Stem}} - 1 \right].\tag{6}$$

Measurements of the isotope ratio of total water extracted from leaves (R_l) were also expressed relative to stem water using equation (6).

We compared predictions of the evaporative enrichment model (expressed as Δ_E using equation (6)) to measurements of the oxygen isotope composition of total water extracted from plant leaves (expressed as Δ_L using equation (6)). Bulk leaf water should not be as enriched in ^{18}O as the water at the evaporative sites because of gradients in the isotope composition of water in plant leaves. The gradients are predicted to result from a shifting balance between the bulk flow of unfractionated water into the leaf and the back diffusion of heavy isotope molecules away from the sites of evaporative enrichment. The model of Farquhar and Lloyd (1993, 1994) was used to relate the isotopic ratio of total foliage water (Δ_L) to that at the evaporative sites within leaves (Δ_E) as shown below:

$$\Delta_L = \frac{CD}{EL} \Delta_E (1 - e^{-EL/CD}),\tag{7}$$

where C is the molar concentration of water ($5 \cdot 55 \times 10^4$ mol m^{-3}); D is the diffusivity of $H_2^{18}O$ ($2 \cdot 66 \times 10^{-9}$ m^2 s^{-1} in water (Wang 1954)); E is the transpiration rate (mol m^{-2} s^{-1}); and L is the effective pathlength (m), averaged over the total leaf, for movement of water from leaf xylem veins (unfractionated) to the sites of evaporative, isotopic enrichment.

Oxygen isotope exchange reaction

In the chloroplast, carbonic anhydrase catalyses the hydration of CO_2 and dehydration of HCO_3^- to facilitate the diffusion of CO_2 to the sites of carboxylation. It is assumed that carbonic anhydrase activity acts to fully equilibrate CO_2 with chloroplast water. At full isotopic equilibrium, CO_2 in the chloroplast will have the following composition:

$$R_c = R_w \alpha_{bc}, \tag{8}$$

where α_{bc} is the equilibrium CO_2–H_2O exchange isotope effect at the leaf temperature (Bottinga and Craig 1969), and R_w is the isotopic composition of chloroplast water (which is assumed to be equal to that of the water at the evaporation sites, R_e, as described by equation (5)). The $^{18}O/^{16}O$ ratio of CO_2 in the chloroplast (R_c) is expressed relative to that of the oxygen isotope composition of atmospheric CO_2 (R_a), as shown below:

$$\Delta_{ca} = \left[\frac{R_c}{R_a} - 1\right]. \tag{9}$$

Discrimination during photosynthetic gas exchange

Discrimination against $C^{18}O^{16}O$ during photosynthetic gas exchange is described by the following equation (Farquhar and Lloyd 1993, 1994):

$$\Delta C^{18}O^{16}O = \frac{\bar{a} + \dfrac{c_c}{c_a - c_c} \Delta_{ca}}{1 - \dfrac{c_c}{c_a - c_c} \Delta_{ca}}. \tag{10}$$

The CO_2–H_2O exchange reaction in the chloroplast determines the isotopic composition of CO_2 in the chloroplast (R_c), which is expressed relative to the isotopic composition of atmospheric CO_2 (R_a) in the term Δ_{ca} (equation (9)). The influence of the oxygen isotopic composition of chloroplast CO_2 on discrimination by the leaf depends on how much CO_2 diffuses out of the leaf after equilibrating with chloroplast water. The term $(c_c/(c_a - c_c))$ defines the amount of CO_2 that diffuses out of the leaf. The term $\bar{a}$ describes the net fractionation during diffusion of CO_2 into and out of the leaf.

The isotopic equilibrium between CO_2 and water in the chloroplast may not be complete, depending on the relative activities of carbonic anhydrase and Rubisco. Equation (10) can be modified to account for incomplete isotopic equilibrium as shown below (Farquhar and Lloyd 1993, 1994):

$$\Delta C^{18}O^{16}O = \frac{\bar{a}(1 + 3\varrho) + \dfrac{c_c}{c_a - c_c}(\Delta_{ca} + 3\varrho b)}{1 - \dfrac{c_c}{c_a - c_c} \Delta_{ca} + 3\varrho \dfrac{c_c}{c_a - c_c}}, \tag{11}$$

where ϱ is the ratio of the rate of carboxylation by Rubisco to the rate of hydration of CO_2 by carbonic anhydrase, and b represents fractionation against $C^{18}O^{16}O$ during carboxylation. The factor 3 occurs because HCO_3^- has three times as many oxygen atoms as water.

Including the effects of dark respiration and photorespiration, equation (11) can be modified as shown below (Farquhar and Lloyd 1994):

$$\Delta C^{18}O^{16}O = \frac{\bar{a}(1 + 3a^*) + \dfrac{c_c}{c_a - c_c}(\Delta_{ca} + 3\varrho b + 3m\Delta_{mc})}{1 - \dfrac{c_c}{c_a - c_c} \Delta_{ca} + 3a^* \dfrac{c_c}{c_a - c_c}}, \tag{12}$$

where a^* is the ratio of the rate of assimilation to the rate of hydration of CO_2 by carbonic anhydrase, Δ_{mc} is $R_m/R_c - 1$, the difference in isotopic composition between CO_2 evolved by the mitochondria and that in the chloroplast and m is the ratio of mitochondrial CO_2 evolution to hydration.

If carbonic anhydrase activity is low, and the rates of hydration of CO_2 and dehydration of HCO_3^- are slow relative to the rate of carboxylation catalysed by Rubisco, equations (11) and (12) can be reduced to the following:

$$\Delta C^{18}O^{16}O = \bar{a}\frac{c_a - c_c}{c_a} + b\frac{c_c}{c_a},\tag{13}$$

which is analogous to that for $^{13}CO_2$ discrimination (Farquhar *et al.* 1989).

Results

When exposed to progressively higher vapour pressure deficits, *P. vulgaris* leaves showed the expected decline in intercellular/ambient CO_2 ratios and a correlated decline in carbon isotope discrimination (Fig. 1) as predicted by theory (Farquhar *et al.* 1989). In addition isotopic discrimination during transpiration ($\Delta H_2^{18}O$) increased as expected when leaves were exposed to higher vapour pressure deficits. In contrast to $\Delta^{13}CO_2$, there was an increase in $\Delta C^{18}O^{16}O$ as leaf–air vapour pressure difference was increased, and this was positively correlated with the change in leaf water ^{18}O composition (Fig. 1).
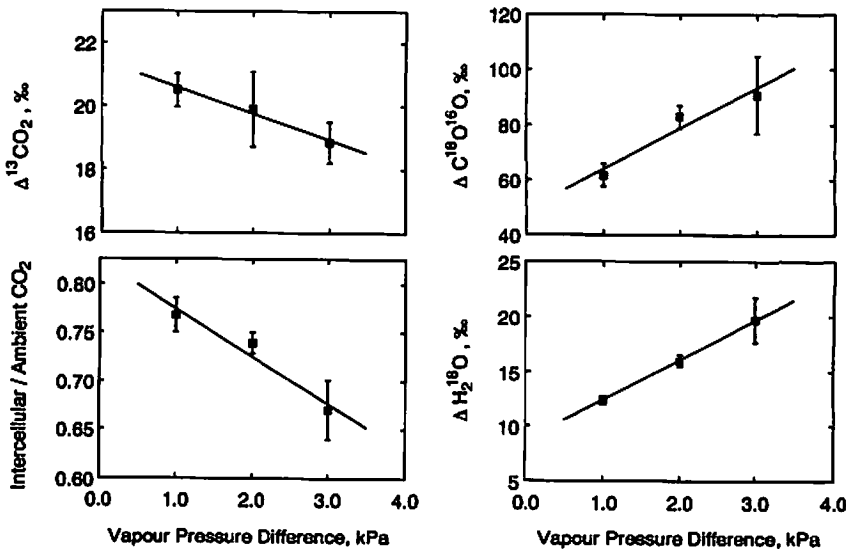

Fig. 1. The effect of changes in the leaf–air vapour pressure difference on the ratio of intercellular CO_2/ambient CO_2, the carbon and oxygen isotopic discrimination during on-line CO_2 assimilation measurements ($\Delta^{13}CO_2$, $\Delta C^{18}O^{16}O$), and total leaf water oxygen isotopic discrimination ($\Delta H_2^{18}O$) in *Phaseolus vulgaris*. Data points represent means ± one standard deviation, $n = 3$. Environmental conditions during measurements were: light intensity (400–700 nm), 1600 μmol m^{-2} s^{-1}; leaf temperature, 30°C; stem water $\delta^{18}O_{SMOW} = -15 \cdot 3 \pm 0 \cdot 2‰$, $n = 9$; atmospheric water vapour $\delta^{18}O_{SMOW} = -14 \cdot 7 \pm 0 \cdot 7‰$, $n = 9$; isotopic composition of CO_2 entering the leaf chamber, $\delta^{13}C_{PDB} = -8 \cdot 69 \pm 0 \cdot 17‰$, $n = 14$, $\delta^{18}O_{SMOW} = -3 \cdot 63 \pm 0 \cdot 35‰$, $n = 14$.

The ^{18}O content of bulk leaf water increased in leaves exposed to high vapour pressure deficits as predicted by the evaporative enrichment model (Flanagan *et al.* 1991*b*). The observed leaf water isotopic discrimination ($\Delta H_2^{18}O$), however, was less than that predicted by the evaporative enrichment model (Table 1). The extent of the difference between observed and modelled $\Delta H_2^{18}O$ values increased as the vapour pressure difference increased (Table 1). The difference between the isotopic composition of water extracted from an entire leaf and that predicted by the model may result from a shifting balance between the bulk flow of unfractionated liquid water into the leaf (convection) and the back diffusion of heavy isotope molecules away from the evaporative sites within leaves (Farquhar and Lloyd 1993, 1994). The transpiration rate of a leaf and the path length for water movement through a leaf are factors that will determine the magnitude of the difference between calculated isotopic discrimination at the sites of evaporative enrichment and the measured total leaf water isotopic discrimination (Farquhar and Lloyd 1993, 1994). A graphical presentation of the convection–diffusion model of mixing of isotopes in leaf water is shown in Fig. 2. The top panel shows the fractionational difference between modelled and observed leaf water $\Delta H_2^{18}O$ values plotted as a function of transpiration rate for data obtained from experiments illustrated in Fig. 1 and Table 1. The bottom panel shows data collected in separate experiments with *P. vulgaris* by Flanagan *et al.* (1991*b*). The solid lines in Fig. 2 represent predictions of the convection–diffusion model (equation (7)) with path length values of 8·5 mm (top panel) and 6·25 mm (bottom panel). The observed data are consistent with the convection–diffusion model predictions suggesting that water at the evaporative sites within leaves has an isotopic composition close to that predicted by the evaporative enrichment model.

Table 1. The effect of changes in leaf–air vapour pressure difference (VPD, kPa) on oxygen isotopic discrimination during transpiration ($\Delta H_2^{18}O$, ‰) in *Phaseolus vulgaris*

The column labelled 'whole leaf' represents measurements made on total water extracted from leaves. The column labelled 'evaporative sites' represents values calculated with equations (5) and (6). The column labelled 'chloroplast water' represents calculations done using measurements of $\Delta C^{18}O^{16}O$ and equation (10), assuming there was complete isotopic equilibrium between oxygen in CO_2 and oxygen in water. The calculations with equation (10) used values of c_c estimated using equations (14) and (15), using four different combinations of estimates for the fractionation factors (‰) b and f (see Table 2). The environmental conditions during gas exchange measurements are listed in Fig. 1. Values are the mean ± standard deviation, $n = 3$

VPD		Oxygen isotopic discrimination ($\Delta H_2^{18}O$, ‰)				
	Whole leaf	Evaporative sites		Chloroplast water		
			$b = 27·5$ $f = 7$	$b = 27·5$ $f = 0$	$b = 29$ $f = 7$	$b = 29$ $f = 0$
1·05	12·3 ± 0·4	15·5 ± 0·9	11·8 ± 1·0	15·5 ± 1·1	16·4 ± 1·2	20·2 ± 1·3
2·00	15·9 ± 0·5	20·7 ± 0·5	16·5 ± 8·6	21·9 ± 9·4	23·2 ± 9·3	28·8 ± 10·2
3·00	19·7 ± 2·0	25·7 ± 0·2	22·3 ± 6·2	29·0 ± 6·9	29·6 ± 7·2	36·9 ± 8·2

The observed $\Delta^{13}CO_2$ values in *P. vulgaris* increased with an increase in intercellular/ ambient CO_2 ratios as predicted by theory. However, the observed values were less than predicted $\Delta^{13}CO_2$ values for all observed intercellular ambient CO_2 ratios (Fig. 3). The differences between observed and theoretical carbon isotope discrimination values were likely a result of limitation imposed by CO_2 transfer conductance from the leaf intercellular air spaces to the chloroplast, and the resulting difference between intercellular and chloroplast CO_2 partial pressures (Caemmerer and Evans 1991; Lloyd *et al.* 1992). Ignoring the influence of dark respiration, it is possible to use measured $\Delta^{13}CO_2$ values (Δ_{obs}) and concurrently measured gas exchange characteristics to estimate the CO_2 partial pressure in the chloroplast using the following equations:

$$\frac{c_c}{c_a} = \frac{c_i}{c_a} - \frac{\Delta_i - \Delta_{obs} - f\frac{\Gamma^*}{c_a}}{b - a_w},\tag{14}$$

where

$$\Delta_i = a_b\frac{c_a - c_s}{c_a} + a\frac{c_s - c_i}{c_a} + b\frac{c_i}{c_a}.\tag{15}$$

The symbols Δ, a, a_b, a_w, b are defined above but now refer to discrimination against $^{13}CO_2$, rather than discrimination against $C^{18}O^{16}O$, f is the fractionation with respect to average carbon composition associated with photorespiration, and Γ^* is the CO_2 partial pressure at the compensation point in the absence of respiration during the day. The values used in the calculations were, a (4·4‰), a_b (2·9‰), and a_w (1·8‰) and values for Γ^* were calculated from the regression equation listed by Brooks and Farquhar (1985). Calculations were done with two different values of b (27·5‰, 29‰) and f (0‰, 7‰) (Rooney 1988; Lloyd *et al.* 1992). The chloroplast CO_2 partial pressures, calculated using the above equations, were used for interpreting $\Delta C^{18}O^{16}O$ measurements.
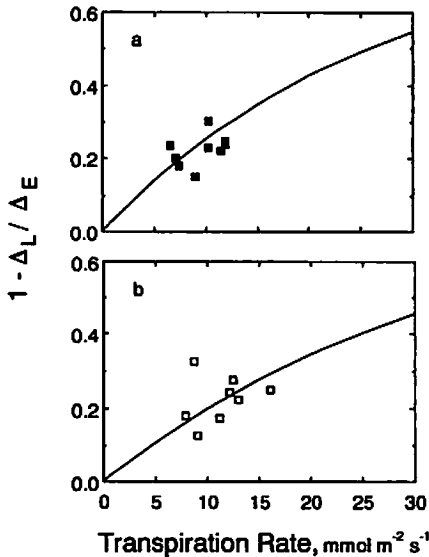

Fig. 2. The influence of transpiration rate on the fractional difference between the modelled (Δ_E) and observed (Δ_L) leaf water oxygen isotopic discrimination in *Phaseolus vulgaris*. The data shown in panel (*a*) are from experiments reported here, and the data in panel (*b*) are from similar experiments reported by Flanagan *et al.* (1991*b*). The modelled values were calculated with equations (5) and (6) for water at the evaporative sites within leaves. The lines represent calculations done with equation (7), using different values for the effective pathlength for water movement (panel (*a*) the L value was: 8·5 mm, panel (*b*) the L value was 6·25 mm).
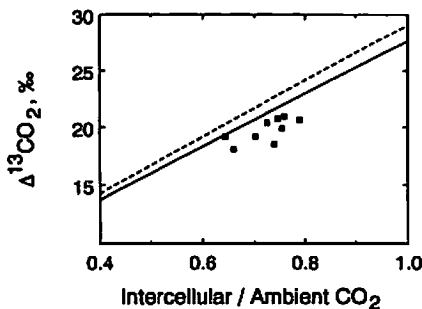

Fig. 3. The effect of changes in the ratio of intercellular CO_2/ambient CO_2 on carbon isotopic discrimination in *Phaseolus vulgaris*. The lines Represent the simple theoretical relationship,

$$\Delta^{13}CO_2 = a\frac{c_a - c_i}{c_a} + b\frac{c_i}{c_a},$$

with values of $a = 4·4‰$ and $b = 27·5‰$ for the solid line, and $a = 4·4‰$ and $b = 29‰$ for the broken line (Farquhar *et al.* 1989; Lloyd *et al.* 1992). The environmental conditions during gas exchange measurements are listed in Fig. 1.

Table 2. The influence of fractionation factor estimate on calculated values of the partial pressure of CO_2 in the chloroplast (c_c) and modelled values of discrimination against $C^{18}O^{16}O$ ($\Delta C^{18}O^{16}O$) for *Phaseolus vulgaris* and *Senecio* spp.

The values of b (fractionation by Rubisco against $^{13}CO_2$) and f (fractionation with respect to average carbon composition associated with photorespiration) were altered in equations (14) and (15) and the effect on calculated c_c was determined. The value of c_c is expressed as the difference ($c_i - c_c$), for intercellular CO_2 partial pressure (c_i) measured at an ambient CO_2 of approximately 35 Pa. The calculated c_c values were then used in equation (10) to calculate $\Delta C^{18}O^{16}O$. Average values (±standard deviation, $n = 9$) are shown for both species. The environmental conditions during gas exchange measurements are listed in the captions to Figs 1 and 4

b (‰)	f (‰)	*P. vulgaris* ($c_i - c_c$) (Pa)	*Senecio* spp. ($c_i - c_c$) (Pa)	*P. vulgaris* $\Delta C^{18}O^{16}O$ (‰)	*Senecio* spp. $\Delta C^{18}O^{16}O$ (‰)
27·5	7	0·7 ± 1·2	5·5 ± 1·1	97 ± 20	89 ± 16
27·5	0	1·9 ± 1·2	6·7 ± 1·1	82 ± 17	76 ± 13
29	7	2·1 ± 1·2	6·8 ± 1·0	80 ± 16	75 ± 11
29	0	3·2 ± 1·2	7·9 ± 1·0	70 ± 14	65 ± 9

There was good agreement between the observed $\Delta C^{18}O^{16}O$ values and the modelled predictions of equation (10) for both *P. vulgaris* and *Senecio* spp. (Fig. 4). The modelled predictions of $\Delta C^{18}O^{16}O$, however, were sensitive to the values used for the fractionation factors b and f (Table 2). An increase in the value of b, while f remained constant, resulted in a lower value of c_c and a lower predicted $\Delta C^{18}O^{16}O$ value. In contrast, an increase in f, while b remained constant, resulted in a higher c_c and higher predicted $\Delta C^{18}O^{16}O$ value (Table 2). Good correlation between observed and modelled $\Delta C^{18}O^{16}O$ values was obtained with $b = 27·5‰$, $f = 0‰$ for both *P. vulgaris* and *Senecio* spp. (Fig. 4).
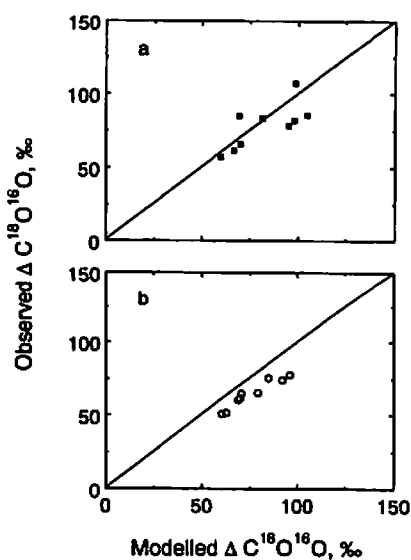

Fig. 4. Comparison of modelled and observed $C^{18}O^{16}O$ discrimination. Complete isotopic equilibrium between oxygen in CO_2 and chloroplastic water was assumed for model calculations. The isotopic composition of water in the chloroplast was assumed to be equal to that at the evaporative sites within leaves, calculated using equation (5). (*a*) *Phaseolus vulgaris*, modelled $C^{18}O^{16}O$ discrimination values were calculated with c_c values estimated using $b = 27·5$, $f = 0$ in equations (14) and (15). Environmental conditions are listed in the caption to Fig. 1. (*b*) *Senecio* spp., modelled $C^{18}O^{16}O$ discrimination values were calculated with c_c values estimated using $b = 27·5$, $f = 0$ in equations (14) and (15). Environmental conditions during measurements were: light intensity (400–700 nm), 1600 μmol m^{-2}, s^{-1}; leaf temperature, 25°C; leaf–air vapour difference ranged from 1·44 to 1·67 kPa; source water $\delta^{18}O_{SMOW} = -10·0 \pm 0·1‰$, $n = 2$; isotopic composition of CO_2 entering the leaf chamber, $\delta^{13}C_{PDB} = -35·02 \pm 0·03‰$, $n = 9$, $\delta^{18}O_{SMOW} = 10·20 \pm 0·09‰$, $n = 9$.

Calculations of $\Delta C^{18}O^{16}O$ using equation (10) were done assuming that the oxygen isotopic composition of water in chloroplasts was the same as the isotopic composition of water at the evaporative sites within leaves as predicted by equation (5). This appeared to be a good assumption because back calculations of the isotopic composition of chloroplast water (R_w and associated $\Delta H_2^{18}O$ values), from observed $\Delta C^{18}O^{16}O$ values and concurrently measured gas exchange characteristics using equations (8) and (10), were more closely related to the isotopic composition of water at the evaporative sites (R_e) than to that of bulk leaf water (Table 1). However, the calculation of the isotopic composition of chloroplast water from measured $\Delta C^{18}O^{16}O$ values was dependent on the values used for the fractionation factors b and f (Table 1).

Discussion

As observed in several previous studies, the Craig and Gordon (1965) evaporative enrichment model overestimates the degree of isotopic enrichment observed in whole leaves (Table 1; Dongmann *et al.* 1974; Allison *et al.* 1985; Leaney *et al.* 1985; Bariac *et al.* 1989; Walker *et al.* 1989; Walker and Brunel 1990; Yakir *et al.* 1990; Flanagan *et al.* 1991*a*, 1991*b*; Walker and Lance 1991), and the possible factors contributing to this difference have been previously discussed (Farquhar and Lloyd 1993, 1994; Flanagan 1993). Because the Craig–Gordon (1965) model predicts the isotopic composition of water at the evaporative sites, it is expected to overestimate the isotopic enrichment of whole leaf water. The isotopic composition of whole leaf water should represent a continuum from unfractionated water in leaf xylem tissue to water maximally enriched in heavy isotopes at the sites of evaporation within leaves. Such a continuum of water with progressively different isotopic compositions has been observed by Yakir *et al.* (1989), when they sampled different aliquots of water pushed out of a leaf by a pressure chamber. The convection–diffusion model of Farquhar and Lloyd (1993, 1994) predicts that the difference between whole leaf water isotopic composition and that calculated by the evaporative enrichment model should vary with transpiration rate and the effective path length for water movement through leaves. Data presented in this paper and similar experiments conducted by Flanagan *et al.* (1991*b*) suggest that the effective path length for water movement in *P. vulgaris* leaves is approximately $6\cdot0$–$9\cdot0$ mm. This effective path length will be much larger than direct measurements of leaf anatomical properties (i.e. distance from vein to intercellular air space), because of the complex movement of water through leaves to the sites of evaporation. Equation (7) is also affected by heterogeneity in gas exchange characteristics across the leaf surface which results in an overestimation of the effective path length (Farquhar and Lloyd 1994). Flanagan *et al.* (1993) calculated the effective path length to be 22 mm for foliage in a xylem-tapping mistletoe (*Phoradendron juniperinum*), and 27 mm in the host tree species (*Juniperus osteosperma*) based on measurements of gas exchange and leaf water isotopic discrimination under field conditions, where within-leaf heterogeneity in gas exchange characteristics was likely.

It is important to know the oxygen isotopic composition of chloroplast water, within the heterogeneous mixture of total leaf water, in order to predict the extent of $C^{18}O^{16}O$ discrimination during photosynthetic gas exchange. Yakir *et al.* (1993) have measured the isotopic composition of oxygen evolved during photosynthesis in order to directly determine the oxygen isotopic composition of chloroplast water. Such an approach is based on the observation of no isotopic fractionation during the water splitting reaction of photosystem II (Guy *et al.* 1993). The results obtained by Yakir *et al.* (1993) indicated that chloroplast water is less enriched in ^{18}O than bulk leaf water. In contrast our results, based on back calculation from observed $\Delta C^{18}O^{16}O$ values and associated gas exchange measurements, suggest that the oxygen isotopic composition of chloroplast water is more similar to that of water at the evaporative sites within leaves and is enriched above that of bulk leaf water, although our calculations are dependent on assumptions about the fractionation

factors b and f (Table 1). Further work is required to determine the oxygen isotopic composition of chloroplast water during photosynthesis under a range of environmental conditions.

There was good agreement between observed and modelled $\Delta C^{18}O^{16}O$ values (Fig. 4). The modelled $\Delta C^{18}O^{16}O$ values were calculated assuming the oxygen isotopic composition of water in the chloroplasts was the same as the isotopic composition of water at the evaporative sites, and by assuming there was complete isotopic equilibrium in the chloroplast between oxygen in CO_2 and oxygen in water. Full isotopic equilibrium between CO_2 and water in the chloroplast would require a large amount of carbonic anhydrase and could be very costly in terms of nitrogen invested in the protein (Cowan 1986). Several studies have indicated, however, that leaves of many C_3 plants have high carbonic anhydrase activity (Reed and Graham 1980; Makino *et al.* 1992). In addition, the activity of carbonic anhydrase may be decreased quite substantially (15%) before CO_2 assimilation is inhibited (Edwards and Mohamed 1973; Randall and Bouma 1973; Jacobson *et al.* 1975). Six of the nine observed $\Delta C^{18}O^{16}O$ values for *P. vulgaris* were lower than values predicted by equation (10) (Fig. 4). The observed values for those six points are consistent with incomplete isotopic equilibrium between chloroplast CO_2 and water. Estimates of ϱ, the ratio of the rate of carboxylation by Rubisco to the rate of hydration of CO_2 by carbonic anhydrase, were made by adjusting ϱ values in equation (11) until predicted $\Delta C^{18}O^{16}O$ values matched observed values. These calculations were done for three values of discrimination by Rubisco against $C^{18}O^{16}O$, 0‰, 15‰ and 30‰, resulting in ϱ values of $0 \cdot 013 \pm 0 \cdot 008$, $0 \cdot 016 \pm 0 \cdot 009$ and $0 \cdot 019 \pm 0 \cdot 011$ (means $\pm$ standard deviation, $n = 6$), respectively. Adjusting the value of the fractionation factors used to calculate c_c and $\Delta C^{18}O^{16}O$ in Fig. 4 from $b = 27 \cdot 5$, $f = 0$ to $b = 27 \cdot 5$, $f = 7$ resulted in eight observed $\Delta C^{18}O^{16}O$ values below that predicted by equation (10). Calculation of ϱ in equation (11) for three values of discrimination by Rubisco against $C^{18}O^{16}O$, 0‰, 15‰ and 30‰, resulted in values of $0 \cdot 025 \pm 0 \cdot 012$, $0 \cdot 029 \pm 0 \cdot 014$ and $0 \cdot 036 \pm 0 \cdot 016$ (means $\pm$ standard deviation, $n = 8$), respectively. These results suggest that Rubisco catalysed carboxylation occurs at only 1–3% of the rate of carbonic anhydrase catalysed hydration/dehydration of CO_2. The carbon dioxide in the chloroplast should, therefore, be at or very near oxygen isotopic equilibrium with chloroplast water.

The good agreement between modelled and observed $\Delta C^{18}O^{16}O$ values is encouraging (Fig. 4), however several uncertainties remain to be investigated further. The modelled values of $\Delta C^{18}O^{16}O$ are strongly dependent on a non-linear relationship with c_c, the partial pressure of CO_2 in the chloroplast (Farquhar *et al.* 1993). A large increase in the modelled $\Delta C^{18}O^{16}O$ value (typically 30–35‰ in the conditions used for our experiments) occurs with a change in the ratio of chloroplast CO_2 to ambient CO_2 from $0 \cdot 45$ to $0 \cdot 65$, a range of variation in c_c/c_a that may regularly occur in C_3 plants. An error or uncertainty in the value of c_c can cause a large change in the predicted value of $\Delta C^{18}O^{16}O$. The techniques used to estimate c_c in our experiments are strongly influenced by heterogeneities in leaf gas exchange properties (Lloyd *et al.* 1992). Non-uniform stomatal closure and spatial variation in leaf photosynthetic gas exchange properties may be a common occurrence when leaves are exposed to low humidities (Mott *et al.* 1993), as was done in our experiments with *P. vulgaris*. The calculations of c_c are also dependent on the values of fractionation factors b and f used in equations (14) and (15) (Table 2), although the values we used for b and f in Fig. 4 are reasonable (Farquhar *et al.* 1989; Lloyd *et al.* 1992). The uncertainties described above set constraints on the confidence that can be placed on calculated estimates of ϱ, the activity ratio of Rubisco to carbonic anhydrase in the chloroplast, and the oxygen isotopic composition of chloroplast water, calculated from observed $\Delta C^{18}O^{16}O$ values and concurrently measured gas exchange characteristics.

Knowledge of the physiological processes involved in $C^{18}O^{16}O$ discrimination may contribute to an improved understanding of the global carbon cycle. Farquhar *et al.* (1993) have used the leaf level $C^{18}O^{16}O$ discrimination model (equation (10)) to develop a global model that predicts the influence of terrestrial vegetation on the oxygen isotopic ratio of

atmospheric CO_2. The latitudinal gradient in the ^{18}O value of atmospheric CO_2 observed by Francey and Tans (1987), can be explained by the Farquhar *et al.* (1993) global model of $C^{18}O^{16}O$ discrimination during photosynthetic gas exchange. Friedli *et al.* (1987) have also shown, in theory, that oxygen isotopic exchange with vegetation and soil should be important processes determining the $^{18}O/^{16}O$ ratio of atmospheric CO_2. Since the terrestrial biosphere has a different effect on the oxygen isotopic composition of atmospheric CO_2 than does CO_2 exchange with the oceans (Farquhar *et al.* 1993), long-term monitoring of changes in the oxygen isotopic composition of atmospheric CO_2 may help determine whether the ocean or the terrestrial biosphere is responsible for the current imbalance between sources and sinks of CO_2 in the global carbon budget (Sundquist 1993).

Acknowledgments

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