On the Nature of Carbon Isotope Discrimination in C\textsubscript{4} Species

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Abstract

Theory is developed to explain the carbon isotopic composition of plants with the dicarboxylic acid (C\textsubscript{4}) pathway of carbon fixation. It is shown how leakage of inorganic carbon from the bundle sheath cells to the mesophyll cells can affect carbon isotopic discrimination. It is suggested that different types of C\textsubscript{4} pathways will show variation in the degree of leakiness, reflecting variations in anatomy, and in the requirements for oxygen egress from the bundle sheath cells. It is also suggested that quantum yields may reflect these variations. A simplified expression is developed relating discrimination to leakiness and the ratio of intercellular and atmospheric partial pressures of CO\textsubscript{2}.

Introduction

The \(^{13}\text{C}/^{12}\text{C}\) ratios in higher terrestrial plants fall into three categories which are associated with the pathways of carbon assimilation—the conventional (C\textsubscript{3}) pathway, the dicarboxylic acid (C\textsubscript{4}) pathway, and the pathway associated with crassulacean acid metabolism (CAM). There is also variation within these groupings. In a previous paper (Farquhar et al. 1982b), the causes of variation in the C\textsubscript{3} pathway were discussed, and it was suggested that they may be associated with variations in the ratio of intercellular and atmospheric partial pressures of CO\textsubscript{2}, and hence of changes in water use efficiency.

The purpose of this communication is to provide some understanding of the variation in the \(^{13}\text{C}/^{12}\text{C}\) ratio among plants with the C\textsubscript{4} pathway. A quantitative expression is developed relating the ratio to the proportion of CO\textsubscript{2} produced by decarboxylation in the bundle sheath cells which leaks back into the mesophyll cells, and to the ratio of intercellular and atmospheric \(p(\text{CO}_2)\). The results support earlier suggestions that leakage may be significant (Berry and Farquhar 1978) and that variation in the \(^{13}\text{C}/^{12}\text{C}\) ratio in C\textsubscript{4} species may reflect variations in the amount of leakage (Hattersley 1976).

Theory and Discussion

Discrimination in C\textsubscript{3} Species

Farquhar et al. (1982b) developed an equation relating the carbon isotope composition of C\textsubscript{3} species to the ratio, \(p_i/p_a\), of intercellular and atmospheric partial pressures of CO\textsubscript{2}

\[
\delta = \delta_{\text{env}} - a - (b_3 - a)p_i/p_a ,
\]

where \(\delta\) is the carbon isotopic composition (\%) relative to that in a standard limestone, \(\delta_{\text{env}}\) is the composition (\%) of the CO\textsubscript{2} in the environment, \(a\) is the fractionation (\%) caused by diffusion, and \(b_3\) is the fractionation (\%) caused by ribulose bisphosphate (RuP\textsubscript{2}) carboxylation. In air \(a = 4.4\), and in an aqueous environment \(a \approx 0\). These values are
predictions only and the latter, in particular, needs to be checked experimentally. In C₃
species, $b_3$ is thought to be near 27 and experiments of Farquhar et al. (1982a) are in fair
agreement with equation (1) with $b_3 = 27$.

Equation (1) may be rewritten in a form which makes its basis more easily understood:

$$\delta - \delta_{\text{env}} = -a \frac{P_a - P_i}{P_a} - b_3 \frac{P_i}{P_a}.$$  (2)

If gaseous diffusion causes a large drop in the intercellular $p(CO_2)$, the right-hand side
tends to $-a$ whereas, if the drop is small, it tends to $-b_3$. Equation (2) may be extended
to include the case where the $p(CO_2)$ at the leaf surface, $p_i$, is less than that in the air,
because of significant boundary layer resistance, and where the $p(CO_2)$ at the sites of
carboxylation, $p_c$, is significantly less than that in the intercellular spaces:

$$\delta - \delta_{\text{env}} = -a_b \left( \frac{P_a - P_s}{P_a} \right) - a \left( \frac{P_i - P_s}{P_a} \right) \left( \frac{P_i - P_s}{P_a} \right) - b_3 \frac{P_s}{P_a}.$$  (3)

where $a_b$ ($\%$) is the fractionation occurring during diffusion through the boundary layer,
$1 \cdot 10^3$ is the fractionation occurring at 25°C as $CO_2$ enters solution (Vogel et al. 1970)
and $a_i$ ($\%$) is the fractionation occurring during diffusion through water, thought to be
near zero. From the Pohlhausen analysis (Kays 1966), the ratio of diffusivities in the
boundary layer should be that in still air raised to the power of $\frac{2}{3}$. Since the binary
diffusivity of $^{12}CO_2$ and air is thought to be 120044 times that involving $^{13}CO_2$ and air
(Craig 1954), $^{12}CO_2$ should move 1.0029 times more easily through a boundary layer of
air than $^{13}CO_2$ (Vogel 1980). Thus $a_b = 2.9$. The form of equations (2) and (3) is
analogous to the 'chain of resistances' used by Vogel (1980), although the final term
involving carboxylation is not a resistance linking one region with a $p(CO_2)$ of $p_c$ to
another at zero $p(CO_2)$.

The Bases of Discrimination in C₄ Species

The bases of discrimination against $^{13}C$ in C₄ plants are more complex. $CO_2$ diffuses
through stomata, dissolves, is converted into $HCO_3^-$, and fixed by phosphoenolpyruvate
(PEP) carboxylase into oxalacetate. Various transformations then occur which are different
for the various C₄ types, but the net result in all cases is that $CO_2$ is released in the bundle
sheath cells and refixed by $RuP_2$ carboxylase (Hatch and Osmond 1976). At equilibrium,
the heavier isotope, $^{13}C$, concentrates in $HCO_3^-$ by $7.9\%$ at 25°C with respect to gaseous
$CO_2$; the effect depends on temperature, being $8.5\%$ at 20°C and $7.4\%$ at 30°C (Mook
et al. 1974). In turn, PEP carboxylase discriminates against $H^{13}CO_3^-$ by about $2.2\%$
(O'Leary 1981). The net effect is that of a discrimination against $^{13}C$ of $b_4\%$ with respect
to the $CO_2$ in the intercellular spaces. At 25°C, $b_4 = -5.7\%$, and this fractionation actually
favours $^{13}C$. There is little opportunity for discrimination between PEP carboxylation in
the mesophyll cells and the release of $CO_2$ in the bundle sheath cells, because of the lack
of significant biochemical branches. No further discrimination would occur if the bundle
sheath cells were gas tight (Whelan et al. 1973) but some $CO_2$ and $HCO_3^-$ is likely to leak
out of these cells into the mesophyll cells (Berry and Farquhar 1978) where it mixes with
other $CO_2$ which has diffused in through the stomata. The leak is a branch from the main
path of carbon and allows some discrimination by the $RuP_2$ carboxylase in the bundle
sheath cells. Other complications due to dark and photorespiration are ignored for the
present.

Dark Fixation of $CO_2$ by Crassulacean Acid Metabolism

In the simplest case, where no leakage occurs, discrimination against $^{13}C$ will again be
given by equation (1), but with $b_3$ replaced by $b_4$. Thus

$$\delta = \delta_{\text{env}} - a - (b_4 - a) \frac{P_i}{P_a}.$$  (4)
At sites in the northern hemisphere, $\delta_{\text{env}}$ exhibits seasonal and longer-term variations as the local atmospheric CO$_2$ concentration changes; the mean value is thought to be close to that in the southern hemisphere (Keeling et al. 1979). Goodman (1980) observed no seasonal variation in southern hemisphere maritime air with a mean of $-7.8\%$. This value will probably need to be corrected for N$_2$O contamination, $+0.3\%$ in the northern hemisphere (Keeling et al. 1979).

Substituting for the various parameters in equation (4) the following results, applicable at $25^\circ$C,

$$\delta = -12.2 + 10.1 \frac{p_s}{p_a},$$

which may be useful in predicting the isotopic composition of the C$_4$ of malate formed in the dark by plants with crassulacean acid metabolism (O'Leary and Osmond 1980). O'Leary and Osmond measured values for $\delta$ of $-7.4$ and $-4.6\%$ in the C$_4$ of malate isolated from Kalanchoe daigremontiana and Bryophyllum pinnatum, respectively; these values correspond, according to equation (5), to intercellular $p$(CO$_2$) of 162 and 256 $\mu$bar, respectively. These would represent the mean $p$(CO$_2$), weighted according to the rate of CO$_2$ fixation, if it were not for complications of dark respiration and fumarase activity as discussed by the authors.

The Effect of Leakage from the Bundle Sheath

Troughton et al. (1974) observed a trimodal distribution of $\delta^{13}$C values in terrestrial species; the first group (which included the known C$_4$ species) had a range of $-10$ to $-19$, and a mean of $-13.6\%$. The most negative of these values are from species with crassulacean acid metabolism, and it is likely that some of the carbon was fixed during the day using the normal C$_3$ pathway (Osmond et al. 1973). Nevertheless, it is impossible to account for most of the remaining C$_4$ values using equation (5), and this suggests that C$_4$ bundle sheaths may be somewhat 'leaky' in order to obtain such negative values.

Following Berry and Farquhar (1978), an expression may be written for the leakage per unit leaf area ($L$, $\mu$mol m$^{-2}$ s$^{-1}$) of inorganic carbon species from the bundle sheath:

$$L = g_s(p_s - p_m),$$

where $g_s$ is the conductance to leakage (see Appendix 2), $p_s$ is the $p$(CO$_2$) in equilibrium with the dissolved CO$_2$ in the bundle sheath, and $p_m$ that in the mesophyll cells. While there is no evidence that fractionation is associated with leakage as such, for generality it is assumed in the detailed analysis given in Appendix 1 that the conductance to leakage of $^{13}$CO$_2$/H$^{13}$CO$_3^-$ is $s\%$ less than that for $^{12}$CO$_2$/H$^{12}$CO$_3^-$ This fractionation may differ from $\alpha$, the value associated with diffusion from the intercellular spaces to the sites of PEP carboxylation, and is discussed later.

In Appendix 1, an analysis of fractionation is made, based on the scheme as shown in Fig. 1. It is initially assumed that ‘dark’ respiration occurs in the light in both bundle sheath and mesophyll cells, but that there is no intrinsic fractionation associated with these processes. A similar assumption is initially made about photorespiration in the bundle sheath cells. The net result is that these three processes affect isotopic composition only via their effects on $p_s$, $p_m$, $p_a$, etc. It is also initially assumed that the CO$_2$ in the intercellular spaces is in equilibrium with that dissolved in the cytoplasm, and with the HCO$_3^-$ at the sites of PEP carboxylation. The following expression for isotopic composition results:

$$\delta = \delta_{\text{env}} - \alpha - \frac{p_a - p_l}{p_s} - \frac{p_l - p_1}{p_s} - \frac{b_4}{V_p} \left( \frac{b_3 L p_s/(p_a - p_l) - s L}{V_p + L p_s/(p_a - p_l)} \right) \frac{p_s}{p_a},$$

where $V_p$ is the rate of PEP carboxylation. To relax simultaneously all assumptions listed above would require a more complicated expression for composition. The results of relaxing
Fig. 1. Simplified scheme of fluxes involved in C₄ carbon gain. \( p_a \) ambient \( p(CO_2) \); \( A \), \( CO_2 \) assimilation rate; \( p_i \), intercellular \( p(CO_2) \); \( p_m \), \( p(CO_2) \) at site of phosphoenolpyruvate (PEP) carboxylation; \( c_m \), \( CO_2 \) concentration (mole fraction in solution) equivalent to \( p_m \); \( V_h \), rate of hydration of \( CO_2 \); \( V_d \), rate of dehydration of \( HCO_3^- \); \( H \), Henry constant for \( CO_2 \); \( b_m \), \( HCO_3^- \) concentration (mole fraction in solution) at site of PEP carboxylation; \( \alpha_m \), extent to which \( b_m \) is in equilibrium with \( c_m \) (= \( VJVh \)); \( K_c \), dissociation constant for \( HCO_3^- \); \( b_3 \), \( HCO_3^- \) concentration in bundle sheath cells; \( \alpha_b \), extent to which \( b_b \) is in equilibrium with \( c_b \); \( L_c \), leakage of \( CO_2 \); \( L_b \), leakage of \( HCO_3^- \); \( \phi \), leakage as a proportion of PEP carboxylation, equals proportion of overcarboxylation; \( R_m \), mesophyll respiration rate; \( R_b \), bundle sheath respiration rate; \( V_c \), rate of RuP₂ carboxylation; \( F \), rate of photorespiration.

them singly are as follows. If there is a significant resistance to diffusion of \( CO_2 \) from the intercellular spaces to the mesophyll cytoplasm,

\[
\delta = \delta_{env} - a_b \frac{p_a - p_i}{p_a} - \frac{p_i - p_h}{p_a} - (1 + a_d) \frac{p_i - p_m}{p_a} - \left[ \frac{b_a V_p + b_3 L p / (p_n - p_m) - s L}{V_p + L p m / (p_n - p_m)} \right] p_m / p_a. \tag{7b}
\]

If 'dark' respiration discriminates against \(^{13}C \) by \( e\%o \), and photorespiration by \( f\%o \) (see Appendix 1), \( b_a \) is replaced by \( (b_a - e R_m / V_h) \) and \( b_3 \) by \( (b_3 - [e(R_m + R_b) + f F] / V_h) \) in equation (7a), where \( R_m \) and \( R_b \) are the rates of 'dark' respiration in the mesophyll and bundle sheath compartments respectively, \( F \) is the rate of photorespiration, and \( V_c \) the rate of RuP₂ carboxylation. Even if no such intrinsic discrimination occurs, this adjustment may be required to describe short-term carbon isotopic gain by leaves if substrates are derived from carbon fixed previously, when conditions were different. Because the pool of photorespiratory substrates turns over rapidly (minutes: Morot-Gaudry et al. 1980), it is unlikely to cause complications in this context. If \( CO_2 \) is not in equilibrium with \( HCO_3^- \) in the mesophyll cytoplasm, \( b_a \) is replaced by \( [b_a (1 - V_p / V_h) + (1 + x) V_p / V_h] \), where \( V_h \) is the rate of hydration of \( CO_2 \), and \( x\%o \) is the fractionation associated with hydration (see
Appendix 3). Note that, when the activity of carbonic anhydrase, and hence $V_b$, is large, the expression degenerates to $b_a$.

The proportion of carbon fixed by PEP carboxylation which subsequently leaks out of the bundle sheath is $\phi$, defined by

$$\phi = \frac{L}{V_p}. \tag{8}$$

The 'leakiness', $\phi$, may also be regarded as a measure of the amount of 'overcycling' that occurs in the mesophyll cells in order to raise the $p(CO_2)$ in the bundle sheath cells. The larger $p(CO_2)$ tends to increase carboxylation and decrease oxygenation of RuP$_2$ (Bowes et al. 1971).

Equation (7b) may be rewritten in terms of $\phi$, as follows

$$\delta = \delta_{\text{env}} - a_0 \frac{P_a - P_i}{P_a} - a \frac{P_i - P_1}{P_a} (1 + a) \left( \frac{P_i - P_m}{P_a} \right) \left[ \frac{b_4 + \phi b_3 \frac{P_a - P_m}{P_m} - s}{1 + \phi P_m / (P_a - P_m)} \right] \frac{P_m}{P_a}. \tag{9}$$

The intercellular $p(CO_2)$ in C$_4$ leaves is reported to range between 100 and 200 $\mu$bar (Wong et al. 1979; Boag 1982) and the $p(CO_2)$ at the sites of PEP carboxylation, $p_m$, will be still less. The $p(CO_2)$ in the bundle sheath cells, $p_b$, is likely to be much higher (Hatch 1971), in some cases possibly in excess of 3000 $\mu$bar (Berry and Farquhar 1978). In such cases, this leads to the following approximation of the final, bracketed, term in equation (9):

$$\frac{b_4 + \phi b_3 \frac{P_a - P_m}{P_m} - s}{1 + \phi P_m / (P_a - P_m)} \approx b_4 + (b_3 - s)\phi. \tag{10}$$

The resistance to diffusion of CO$_2$ through the boundary layer is often much less than that through the stomata, and $a_0$ and $a$ are in any case numerically similar. If, further, the resistance to diffusion of CO$_2$/HCO$_3^-$ from the intercellular spaces to the sites of PEP carboxylation is small, and the parameter $s$ is close to zero (see Appendix 3 and discussion by Farquhar et al. 1982b on isotopic effects on the diffusion of inorganic carbon species in aqueous solutions), and the activity of carbonic anhydrase in the mesophyll cytoplasm is sufficient to bring CO$_2$ and HCO$_3^-$ into equilibrium, equation (9) simplifies to

$$\delta = \delta_{\text{env}} - a - (b_4 + b_3 \phi - a) p_i / P_a. \tag{11}$$

Taking $\delta_{\text{env}} = -7.8$, $a = 4.4$, $b_4 = -5.7$ at $25^\circ$C and $b_3 = 27$, equation (11) becomes

$$\delta = -12.2 - (27\phi - 10.1) p_i / P_a. \tag{12}$$

This equation is plotted in Fig. 2 and it is apparent that the leakiness, $\phi$, has a large effect on $\delta$ at high $p_i$ and less at low $p_i$; the effects of changes in $p_i / P_a$ are variable, being zero at $\phi = 0.37$. 

![Fig. 2. $\delta^{13}C$ versus $p_i / P_a$ according to equation (12).](image-url)
While equation (12) necessarily involves simplifications, it is a useful introduction to the more complex equations (7a, 7b) and another version independently developed by Peisker (1983) from the equations of O'Leary (1981).

**Leakiness of the Bundle Sheath in Different C₄ Types**

The importance of leakiness, \( \phi \) (= amount of overcycling in the mesophyll), emerges in the modelling of isotopic fractionation in C₄ species. In this section, possible variation in \( \phi \) between types is assessed on the basis of qualitative differences in bundle sheath permeability.

*\textit{A priori*} there need be no unique relationship between \( \phi \) and the conductance to leakage, \( g_s \). In the extreme case where the \( p(\text{CO}_2) \) in the bundle sheath is sufficiently high to saturate RuP₂ carboxylation, an increase in rate of PEP carboxylation, \( V_p \), would be matched by an equal increase in the absolute amount of \( \text{CO}_2/\text{HCO}_3^- \) leaked from the bundle sheath, \( L \). Because \( L \) is less than \( V_p, \phi = L/V_p \) would increase with no change in \( g_s \), and the increase in rate of decarboxylation (and hence of leakage) would be reflected in the increased gradient \( (p_s-p_a) \).

Nevertheless, in practice there is likely to be coordination between the capacities for PEP and RuP₂ carboxylation minimizing the problem outlined above. Leakage represents a cost to the leaf in terms of both ATP consumption (discussed later) and the production and maintenance of the biochemical machinery for PEP carboxylation and regeneration. Since leaves with a small conductance to leakage through the bundle sheath need less overcycling to maintain a large \( p(\text{CO}_2) \) in the bundle sheath cells, there is likely to be some correlation between \( \phi \) and \( g_s \).

The conductance to leakage is probably substantially reduced in a bundle sheath cell wall which contains a suberized lamella (Hatch and Osmond 1976), as found in many C₄ grasses (Carolin et al. 1973). Hattersley and Browning (1981) found that only the NADP-ME-type and PCK-type C₄ grasses have this structure. It is absent from NAD-ME-type grasses and from all dicots examined so far. On this basis it seems likely that \( \phi \) is greater in dicots and NAD-ME-type grasses than in PCK and NADP-ME type grasses.

Hattersley and Browning support the notion that NADP-ME types are less leaky than the others by pointing out that it is consistent with the differences in nature of the \( \text{CO}_2 \) post-illumination burst as observed by Downton (1970).

A further distinction may be drawn between NADP-ME species and other C₄ types. It appears that NADPH formed via NADP malic enzyme reduces the requirement for photosystem II capacity in this type, and hence the evolution of oxygen (Hatch and Osmond 1976). This means that, for a given conductance to leakage, the \( p(\text{O}_2) \) in the bundle sheath rises less in NADP-ME types. Consequently the \( p(\text{CO}_2) \) required in the bundle sheath to saturate carboxylation and substantially inhibit oxygenation of RuP₂ may be less than in other types, causing less leakage and a smaller value of \( \phi \). It is shown later that the conductance to leakage may be a compromise between the need to reduce leakage of \( \text{CO}_2/\text{HCO}_3^- \) and that of allowing the egress of \( \text{O}_2 \). On this basis NADP-ME species can ‘afford’ to construct their bundle sheaths in a more gas-tight manner, again implying a lower value of \( \phi \).

Summarizing the above arguments, it is likely that the leakiness, \( \phi \), is lowest in NADP-ME type grasses, and greatest for NAD-ME grasses and NAD-ME and PCK dicots, with PCK-type grasses and NADP-ME dicots intermediate.

*Variation in Leakiness of the Bundle Sheath: Estimates from Quantum Yield Data*

Some support for these ideas comes from data on the quantum yield of photosynthesis in C₄ species. To examine these a relationship between quantum yield and leakiness must first be established.

The Calvin cycle requires 2 NADPH and 3 ATP per \( \text{CO}_2 \) fixed, and the mesophyll portion requires 2ATP [although there is uncertainty about the PCK-type (Hatch and
Osmond 1976), making it seem likely that ATP is the limiting metabolite at low irradiances (Berry and Farquhar 1978). The NADPH production required to fix 1 mol CO₂ in turn requires about 8·4 mol quanta (at low irradiances) with a concomitant production of 8 mol H⁺ in linear electron flow (see Farquhar and von Caemmerer 1982 for discussion). Since 3 H⁺ are needed to produce an ATP molecule (Hangarter and Good 1982), another 7 mol H⁺ is needed. If this were also produced by linear flow, whether to NADP⁺, O₂, or some other acceptor, the quantum requirement would then be 15·8, corresponding to a quantum yield of 0·063. However, if the extra H⁺ were produced by the 'traditional' version of cyclic flow, yielding 1 H⁺/hv, the requirement would be 15·4, corresponding to a yield of 0·065.

If leakage occurs, 1 mol of PEP carboxylation and regeneration, which requires 2 mol of ATP and hence 6 mol of quanta, produces only 1−φ mol of RuP₂ carboxylations, in turn requiring 3(1−φ) mol ATP, and 2(1−φ) mol NADPH. The NADPH production requires 8·4(1−φ) mol quanta and supplies enough H⁺ for 8(1−φ)/3 mol ATP, leaving a deficit of (1−φ)/3 mol ATP, requiring (1−φ) mol quanta. Thus (1−φ) mol CO₂ fixed requires 6+9·4(1−φ) mol quanta. To fix 1 mol CO₂ requires [9·4(1−φ)] mol quanta, corresponding to a quantum yield of (1−φ)/(15·4−9·4φ). Denoting the quantum yield as q, the following expression for leakiness results:

\[ \phi_1 = \frac{(1−15·4q)/(1−9·4q)}{ } \]  

Ehleringer and Pearcy (1983) have recently discovered several monocots with quantum yields in excess of 0·65, indicating impossible negative values of leakiness as calculated by equation (13a). Since processes other than CO₂ fixation, including photorespiration (Morot-Gaudry et al. 1980), consume energy and not all quanta absorbed by the leaf are harvested usefully, and since some leakage must also occur, their observations, if correct, suggest that some other, presumably cyclic, process yields 2 (or more) mol H⁺ per mol quanta absorbed, and 2(1−φ) mol ATP per mol quanta absorbed. Such a process was postulated by Mitchell (1976) and other modified cycles with this property have since been suggested (see e.g. Crowther and Hind 1980). If the extra 7 mol H⁺ were produced on such a basis, the minimum quantum requirement would be 11·9, corresponding to a quantum yield of 0·064. If, however, leakage occurs, a quantum yield of (1−φ)/(15·4−9·4φ) results, and the corresponding expression for leakiness is

\[ \phi_2 = \frac{(1−11·9q)/(1−8·9q)}{ } \]  

Ehleringer and Björkman (1977) observed that the leaves of five C₄ dicotyledonous species had a mean yield of 0·054 mol CO₂ fixed per mol quanta observed, corresponding to \( \phi_1 \) and \( \phi_2 \) of 0·34 and 0·69, respectively. Ku and Edwards (1978) found a quantum yield of 0·059 in Zea mays (NADP-ME grass), corresponding to \( \phi_1 = 0·21, \phi_2 = 0·63 \). Robichaux and Pearcy (1980) measured the quantum yield of the shade-adapted Euphorbia forbesii (NADP-ME dicot) to be 0·062, corresponding to \( \phi_1 = 0·11, \phi_2 = 0·59 \). The differences in quantum yield determinations between laboratories could be ascribed to experimental error were it not for other recent data of Ehleringer and Pearcy (1983). These authors have observed systematic variations in quantum yield among C₄ species. The results, together with values of \( \phi_1 \) and \( \phi_2 \) calculated using equations (13a, 13b) are presented in Table 1.

Obviously, equation (13a) will overestimate the actual leakiness at low irradiance, if the H⁺/hv ratio in cyclic flow is 1, as will equation (13b) if the ratio is 2 since, as discussed before, not all the absorbed quanta are used for CO₂ fixation. Photosynthesis in leaves of C₃ species is not as efficient in white light as bundle sheath photosynthesis is assumed to be in the derivation of equation (13). The quantum yields of C₃ plants, with photorespiration suppressed, are typically 0·073–0·08 in white light (Ehleringer and Björkman 1977; Ku and Edwards 1978), and 0·081 in C₃ grasses in the study of Ehleringer and Pearcy (1983), compared to a theoretical maximum of 0·106–0·118. On this basis,
if the 'losses' in C₄ photosynthesis were equal to the apparent 'losses' in C₃ photosynthesis, all calculated values of φ₁, and those of φ₂ except NAD-ME dicots, would be negative in Table 1. It is possible that the variations in Table 1 may reflect variations in non-specific absorption, in photochemical efficiency, or in the degree to which conflicting requirements for ATP and NADPH between the bundle sheath and mesophyll cells are met. Further,

Table 1. Values of leakiness, φ, calculated using equations (13a) and (13b) and from the quantum yield data of Ehleringer and Pearcy (1983)
Quantum yield is calculated as (mol CO₂)/(mol quanta), and data are presented = s.e.m. N, number of species examined

<table>
<thead>
<tr>
<th>C₄ type</th>
<th>N</th>
<th>Quantum yield</th>
<th>Calculated φ</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADP-ME monocots</td>
<td>8</td>
<td>0.065±0.001</td>
<td>-0.03</td>
</tr>
<tr>
<td>PCK monocots</td>
<td>5</td>
<td>0.064±0.002</td>
<td>-0.04</td>
</tr>
<tr>
<td>NAD-ME dicots</td>
<td>6</td>
<td>0.061±0.001</td>
<td>0.14</td>
</tr>
<tr>
<td>NAD-ME monocot</td>
<td>3</td>
<td>0.060±0.001</td>
<td>0.17</td>
</tr>
<tr>
<td>NAD-ME dicots</td>
<td>9</td>
<td>0.065±0.001</td>
<td>0.37</td>
</tr>
</tbody>
</table>

φ at low irradiance may differ from that under normal conditions. Nevertheless, the ranking of calculated φ is in the same order as predicted earlier and does not depend on assumptions about the nature of photophosphorylation. Parenthetically, the ATP requirement of PCK monocots is probably similar to that of NADP-ME and NAD-ME monocots.

Just as quantum yields of C₄ species may reflect variation in φ, Peisker (1983) has suggested that information on leakage may be derived from the oxygen dependence of the CO₂ compensation point.

Comparison with Measured Values of δ¹³C

Unless one accepts that φ may be large, the most difficult observations to explain with the simplified equation (12) are those where δ¹³C is most negative. It should be recognized that anomalies may arise where samples are taken from environments in which δₑᵥₑᵥₑ is rather negative due to substantial soil respiration, human respiration, or to fossil fuel combustion (Lerman 1975; Osmond 1975). Considering, first, values from collections in which the source environments are not stated—Smith and Epstein (1971) observed a value of -18.3% for Atriplex semibaccata and Bender (1971) observed a value of -19.4% for Atriplex rosea. If representative, the latter would mean an overlap with C₃ values since δ¹³C values of -19 can occur in C₃ species when pᵣ is low (Farquhar et al. 1982a). However, δ¹³C for A. rosea has subsequently been reported as -11.1 (Hatch et al. 1972) and -14.3% (Troughton et al. 1974). In the survey of Troughton et al. (1974), which included material from plants grown in controlled environments, the 63 C₄ species had a range of δ¹³C values of -10 to -17.3 and a mean of -13.6%, the most negative being A. buchanii. Note that the most negative values occur in Atriplex species, which are dicotyledonous, and probably more leaky, as discussed before.

Recent surveys give more information about sources, and may be more reliable for the present purposes. Vogel et al. (1978) found values in the range -11.4 to -16.4, with a mean of -13.1% in 28 South African C₄ grass species. Winter (1981) found values in the range -9.9 to -15.6, with a mean of -12.9% in 170 species of the Chenopodiaceae and Polygonaceae collected from the Middle East and USSR. Ziegler et al. (1981) found -10.4 to -13.9, with a mean of -12.4% in 41 desert plants. Hattersley (1982) found -10.6 to -13.5, with a mean of -12.0%, in 31 species of C₄ grasses grown in a well ventilated glasshouse.
Carbon Isotope Discrimination in C₄ Species

Hattersley (1982) found that, although his results were variable, NADP-ME grass species generally had the least negative δ¹³C values, NAD-ME the most negative, with PCK species somewhat intermediate. He also grew three dicots under the same conditions and found that their δ¹³C values were similar to the NAD-ME species. He used equation (11), taking \( p/Na \) as 0.3 and \( b_3 \) as 30, to estimate leakage. With \( b_3 = 27 \), the mean values of \( \phi \) in the various types of grasses become: NADP-ME, 0.27; PCK, 0.34; NAD-ME, 0.43. On the same basis, the mean value of \( \phi \) for the dicots becomes 0.49. It is possible that \( p/Na \) exceeded 0.3 but, using the large value 0.65, the mean values for leakiness are little changed, becoming 0.33, 0.36, 0.40 and 0.43, respectively.

Table 2. δ¹³C and \( p/Na \) values in control and sodium-deficient plants

Leakiness, \( \phi \), calculated according to equation (12), from data of Boag (1982)

<table>
<thead>
<tr>
<th>Species</th>
<th>C₄ type</th>
<th>Treatment</th>
<th>δ¹³C</th>
<th>( p/Na )</th>
<th>( \phi )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Echinochloa crus-galli</td>
<td>NADP-ME</td>
<td>+Na</td>
<td>-11.1</td>
<td>0.65</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Na</td>
<td>-8.0</td>
<td>0.84</td>
<td>0.19</td>
</tr>
<tr>
<td>Eleusine indica</td>
<td>NAD-ME</td>
<td>+Na</td>
<td>-12.1</td>
<td>0.48</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Na</td>
<td>-10.5</td>
<td>0.58</td>
<td>0.27</td>
</tr>
<tr>
<td>Chloris barbata</td>
<td>PCK</td>
<td>+Na</td>
<td>-12.2</td>
<td>0.52</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Na</td>
<td>-10.9</td>
<td>0.69</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Boag (1982) grew three C₄ grasses with and without sodium, measured \( p/Na \) in expanded leaves and then determined δ¹³C. The results are shown in Table 2. Sodium-deficient Echinochloa crus-galli (NADP-ME) had a δ¹³C value of -8.0%, which must be among the least negative values recorded for C₄ species. Note that the calculated value of \( \phi \) is smaller in deficient plants. A possible explanation is that the mesophyll metabolism is affected by the deficiency (C₄ and CAM species require sodium which C₃ species do not: Brownell 1979), and that the RuP₂ carboxylase then draws the bundle sheath \( p(CO₂) \) down to low values (hence reducing \( \phi \)), when the supply of \( CO₂ \) by decarboxylation is reduced. If the supply by decarboxylation is reduced to the extent that the \( p(CO₂) \) in the bundle sheath is not much greater than that in the mesophyll, equations (10), (11) and (12) are invalid. In the limit, when the supply is zero (\( V_p = 0 \)), the 'leakage' is negative, approximately equal to the rate of assimilation, and equation (9) degenerates to that for C₃ leaves (cf. equation (A24) in Appendix 1).

The measurements of δ¹³C, when compared with equation (12), seem to imply that the leakage of \( CO₂ \) from the bundle sheath is typically 30-45%. This is comparable to the \( \phi \) values estimated from the quantum yield data shown in Table 1. However, as discussed earlier, the latter values are probably overestimated, and it is possible that the estimates from equation (12) are also too high. There are various assumptions involved in the derivation of equation (12), apart from that involving the insignificance of \( p_x \) compared to \( p_\alpha \). The value of 27 for \( b_3 \) is based on measurements in material from C₃ species—the relationship between δ¹³C and \( p/Na \) fractionation by RuP₂ carboxylase, and isotopic composition of whole plants grown at high \( CO₂ \) (see Vogel 1980; Farquhar et al. 1982a). More importantly, it is possible that the assumption of equilibrium between \( CO₂ \) and HCO₃⁻ in the mesophyll (and hence that \( [b_4(1-V_p/V_b)+(1+1+x)V_p/V_b] \) may be approximated by \( b_4 \)) may be invalid. The isotope fractionation between gaseous \( CO₂ \) and dissolved HCO₃⁻ of -7.9% at 25°C is an equilibrium value. Cowan (unpublished) argues that, for efficient use of protein, carbonic anhydrase should only be 'invested' at the expense of other proteins, at levels appropriate to the 'return' in terms of increased carboxylation. Such levels may not allow full equilibration at rapid rates of PEP carboxylation. O'Leary (unpublished) has observed that the isotopic exchange of oxygen between \( CO₂ \) and water,
which is facilitated by carbonic anhydrase, is incomplete in several species with crassulacean acid metabolism. If the level of carbonic anhydrase in the mesophyll cytoplasm is insufficient to maintain equilibrium between \( \text{CO}_2 \) and \( \text{HCO}_3^- \), the fractionation will be intermediate between \(-7.9\%\) and the kinetic isotope fractionation associated with the hydration of \( \text{CO}_2 \). The latter value could be large (O'Leary 1981), which would explain the negative values of \( \delta^{13}\text{C} \) without the necessity of invoking substantial leakage. Independent estimates of the isotopic composition of \( \text{HCO}_3^- \) in the mesophyll cytoplasm and of the leakiness of the bundle sheath are needed.

Estimates of the Bundle Sheath Leaksiness from Measurements of Pool Sizes of Metabolites

Hatch and Osmond (1976) estimated the leakiness, \( \phi \), to be less than 10\% from measurements of the relative sizes of the pools of \( \text{C}_4 \) acids and of inorganic carbon. Osmond and Smith (1976) reassessed these estimates. They suggested that the leakage of \( \text{HCO}_3^- \) through the plasmodesmata would be at most 10\% of the \( \text{C}_4 \) acid flux, \( V_p \). They pointed out that \( \text{CO}_2 \) may also diffuse through the plasmalemma and cell wall. They noted that a minimum estimate of the free \( \text{CO}_2 \) concentration in the bundle sheath cells is 25 \( \mu \text{M} \), which could allow further leakage of less than 10\% of \( V_p \). Because \( \text{HCO}_3^- \) is unlikely to be in equilibrium with \( \text{CO}_2 \), the free \( \text{CO}_2 \) concentration is likely to exceed 25 \( \mu \text{M} \) and the total leakage of \( \text{CO}_2/\text{HCO}_3^- \) could easily exceed 20\% of \( V_p \) (i.e. \( \phi > 0.2 \)). Ku and Edwards (1980) estimated the \( \text{CO}_2 \) concentration in \textit{Amaranthus graecizans} as 28 \( \mu \text{M} \).

Apel and Peisker (1978) estimated the conductance to leakage of \( \text{CO}_2 \) from the bundle sheath cells to the mesophyll tissue assuming that the conductance to radial diffusion through an aqueous cylinder is an appropriate analogue. However, of the five species examined, four were NADP-ME monocots and the fifth, \textit{Panicum altissimum} is of an unknown type. The presence of suberized lamellae in the bundle sheath cell walls of NADP-ME monocots means that the analogue used may have been inappropriate (Osmond and Smith 1976).

The mean conductance, \( g_s \), estimated by Apel and Peisker (1978), was 3.1 mmol m\(^{-2}\) s\(^{-1}\) bar\(^{-1}\), obtained using a diffusivity half of that for \( \text{CO}_2 \) in water. Yeoh et al. (1981) measured the Michaelis constant for \( \text{CO}_2 \) of RuP\(_2\) carboxylase from \( \text{C}_4 \) species to be 28-34 \( \mu \text{M} \) \((-1 \text{ mbar} \rho(\text{CO}_2)) \). In the presence of oxygen the Michaelis constant is effectively increased (see Appendix 1) and so a \( \rho(\text{CO}_2) \) in excess of 3 mbar would be needed to saturate the carboxylase. Using the above estimate of \( g_s \) implies a leakage of at least 9 \( \mu \text{mol} \text{ m}^{-2} \text{ s}^{-1} \). With a net assimilation rate of 30 \( \mu \text{mol} \text{ m}^{-2} \text{ s}^{-1} \), this corresponds to a leakiness, \( \phi \), of 0.23. In studies of \textit{Zea mays} (NADP-ME monocot), Mahon et al. (1974) observed release of \( ^{13}\text{CO}_2 \) at 20\% of the rate of fixation of \( ^{14}\text{CO}_2 \). At the time they interpreted this as 'a special form of photorespiration', but it may in part have been leakage.

The Conductance to Leakage of Oxygen

The bundle sheath chloroplasts of some \( \text{C}_4 \) species have very little photosystem II activity, whereas others have abundant activity (Woo et al. 1970; Hatch and Osmond 1976). Raven (1977) noted that, in Gramineae with the NADP-ME pathway of carbon fixation, there is probably very little net \( \text{O}_2 \) production or consumption in the bundle sheath cells. He, and Berry and Farquhar (1978), pointed out that in species with other pathways, the \( \rho(\text{O}_2) \) in the bundle sheath cells may substantially exceed that in the mesophyll cells. This implies that the conductance to leakage must be a compromise between reduced leakage of inorganic carbon and facilitated loss of oxygen, as noted earlier.

In Appendix 2, equations for diffusion of oxygen and inorganic carbon out of the bundle sheath are combined to yield the following expression for leakiness:

\[
\phi = \frac{21 \cdot 15(1+6 \cdot 3\alpha)(\rho_s-\rho_m)}{\Delta \rho_c+21 \cdot 15(1+6 \cdot 3\alpha)(\rho_s-\rho_m)},
\]

(14)
Carbon Isotope Discrimination in $C_4$ Species

where $\beta$ closely approximates the fraction of total leaf oxygen evolution occurring in the bundle sheath cells; $\alpha$ is the extent to which $\text{HCO}_3^-$ equilibrates with $\text{CO}_2$ and has the same path for diffusion; $\Delta p_o$ is the difference in $p(\text{O}_2)$ between the bundle sheath cells and mesophyll cells.

About 50% of the 3-phosphoglycerate produced in the bundle sheath chloroplasts is thought to diffuse to the mesophyll cells, where it is reduced before returning to rejoin the Calvin cycle in the bundle sheath (Hatch and Osmond 1976). This implies that $\beta$ is approximately $0.5$. However $\beta$ is probably smaller in NADP-ME species if NADPH is formed via NADP malic enzyme.

Hatch and Osmond (1976) estimate that the concentration of $\text{CO}_2/\text{HCO}_3^-$ in the bundle sheath of $\textit{Amaranthus edulis}$ (NAD-ME dicot) is 2 mm, which corresponds to a $p(\text{CO}_2)$ of between 60 and 5 mbar, depending on the extent to which $\text{HCO}_3^-$ equilibrates with $\text{CO}_2$. To obtain the lowest bound on the estimate of $\phi$, we take $(p_n-p_m)=5$ mbar, $\alpha=0$. Presumably the $p(\text{O}_2)$ in the bundle sheath cells does not exceed 1 bar, and so $\Delta p_o$ is less than 790 mbar. Substituting these values in equation (14) reveals that $\phi$ must exceed 6%.

It is likely that the inorganic carbon pool is not in equilibrium in bundle sheath cells as the activity of carbonic anhydrase is reported to be low (Graham et al. 1971). Since this pool turns over approximately once per second (Hatch 1971) and the uncatalysed conversion of $\text{CO}_2$ (formed by decarboxylation) to $\text{HCO}_3^-$ is in the range $0.026-0.043$ s$^{-1}$ (Pinseit et al. 196; Eigen et al. 1961), the $p(\text{CO}_2)$ may be at the high end of the range. Taking the $p(\text{CO}_2)$ as 30 mbar, and the $p(\text{O}_2)$ in the bundle sheath as 500 mbar, $\phi$ is then estimated as $0.4$ for this NAD-ME dicot. Further evidence that dicots may be quite leaky comes from the observation that increased $p(\text{O}_2)$ substantially inhibited photosynthesis by $\textit{Amaranthus graecizans}$ (Ku and Edwards 1980).

Fractionation during Leakage

Thus far it has been assumed that the fractionation, $\%$, associated with leakage is close to zero. This would probably be the case if $\text{HCO}_3^-$ levels in the bundle sheath were low (because of low activity of carbonic anhydrase), and most leakage were as $\text{CO}_2$. If, however, $\text{CO}_2$ and $\text{HCO}_3^-$ were in equilibrium, $\text{H}^3\text{CO}_3^-$ would be enriched by $9\%$ and the value of $s$ could be $-6.7$ (at its most negative). On this basis, in the worst case ($s=-6.7$), equation (10) suggests that the values of $\phi$ calculated in this paper may be overestimated by 34/27. To the extent that $\text{HCO}_3^-$, being charged, probably leaks less readily than $\text{CO}_2$, the value of $s$ is more probably closer to zero. A formal treatment of the above is given in Appendix 3. The errors in equation (12) due to the unknown fractionation between $\text{CO}_2$ and $\text{HCO}_3^-$ in the mesophyll are potentially greater.

Growth at High $p(\text{CO}_2)$

In an atmosphere in which the $p(\text{CO}_2)$ is sufficiently large, the gradients of $p(\text{CO}_2)$ in a leaf will become relatively small compared to the absolute values, which will all approach $p_a$. The approximations made in equation (10) (and hence implicit in subsequent equations) become invalid. Instead, in the limit, equation (7a), with modifications discussed before equation (8), becomes

$$\delta = \delta_{\text{env}}-b_3+eR_d/V_c ,$$

(15)

where $R_d (= R_m+R_a)$ is the total rate of 'dark' respiration in the light. The same limiting equation (15) should hold for $\text{C}_3$, $\text{C}_4$ and algal species. Presently available evidence, summarized by Farquhar et al. (1982b), suggests that $e$, the intrinsic fractionation associated with 'dark' respiration, is small. Thus, at sufficiently large $p(\text{CO}_2)$, the full fractionation of RuP$_2$ carboxylase should be expressed in $\text{C}_3$, $\text{C}_4$ and algal species.

Smith and Boutton (1981) grew $\textit{Zea mays}$ plants in a range of CO$_2$ concentrations and found $\delta$ decreased linearly from $-12$ to $-49\%$ as CO$_2$ concentration increased from 0.03
to 5%. However the seedlings were not germinated under these conditions, and the isotopic composition of the commercial CO₂ used for enrichment was not measured (Smith, personal communication). Vogel (1980) avoided these problems and grew algae, *Lycopersicon esculentum* (C₃), and *Zea mays* (C₄ NADP–ME monocot) at increasing levels of CO₂ and examined the discrimination (= δₑᵥₑ – δ) in the resulting plant material. The discrimination tended to a maximum value of approximately 27% in the algae and in *L. esculentum*. In the *Z. mays* plants it varied from 3.3% (implying φ = 0.24) in normal air to 16.5% in air with 1.5% CO₂, but in this case Vogel noted that there was no indication that maximum discrimination had been reached. The gradient of p(CO₂) between the bundle sheath and the mesophyll cells of *Z. mays* is likely to be of the order of 3 mbar, which is not insignificant compared to 15 mbar (1.5% CO₂). Further, the gradient across the epidermis may also be of the order of 1 mbar under these conditions. A much higher ambient p(CO₂) is needed to make these gradients small in comparison to pₛ and hence to estimate b₃ for C₄ species using equation (15).

**On the Application of Equation (11)**

The simplified expression for fractionation in C₃ species [equation (1)] appears to be in fair agreement with experiment (Farquhar et al. 1982a; Bradford et al. 1983). It has been used to interpret the carbon isotopic composition of tree rings (Farquhar 1980; Francey and Farquhar 1982) and to estimate the CO₂ concentration inside an alga grown at high CO₂ levels with non-limiting nitrogen nutrition (Beardall et al. 1982); it has also been used to estimate relative water use efficiencies in mutant and wild-type tomato seedlings (Bradford et al. 1983).

The analogous expression for C₄ species, equation (11), is unlikely to be as useful, since variation in fractionation can be due to changes in both pₗ/pₛ and leakiness. If the activity of carbonic anhydrase in the mesophyll cytoplasm proves to be limiting, equation (11) will have to be replaced by

\[ δ = δₑᵥₑ - a - [b₃(1 - V_p/Vₗ) + (1 + x)]V_p/Vₗ + b₃φ - a]pₗ/pₛ , \]

which is even less manageable. Equation (11) may, however, still find applications in studies of quantum yield, where \( V_p \) is small and \( pₗ/pₛ \) can be estimated independently, using gas-exchange techniques. It may also be adapted for use in algae where bicarbonate transport occurs. It is unknown whether the mechanism involving charge transport is sensitive to isotopic differences. Assuming none, the appropriate value for \( b₄ \), the fractionation relative to gaseous CO₂, is −7.9% at 25°C.

In all of the above it has been assumed that fractionations caused by RuP₂ and PEP carboxylases and carbonic anhydrase are independent of species. The possibility is recognized that some of the variability in carbon isotope composition may be due to natural variation in these properties.

**Acknowledgments**

The author wishes to thank the following for valuable discussions: Drs J. Berry, S. Boag, S. von Caemmerer, I. Cowan, G. Edwards, J. Ehleringer, M. Hatch, P. Hattersley, M. O'Leary, R. Pearcy, J. Raven, A. and B. Smith, J. Vogel and J. White; and Brian Weir for assistance in preparation of the manuscript.

**References**


Carbon Isotope Discrimination in \( \text{C}_4 \) Species


Appendix 1.

**Derivation of an Expression for Carbon Isotope Discrimination in \( \text{C}_4 \) Species**

The equation for diffusion of \( ^{12}\text{CO}_2 \) from the atmosphere to the intercellular spaces is

\[
A = \frac{(p_a - p_i)/P}{r_b + r_i}, \tag{A1}
\]

where \( A \) is the rate of \( ^{12}\text{CO}_2 \) assimilation, \( p_a \) and \( p_i \) are the ambient and intercellular partial pressures of \( \text{CO}_2 \), respectively, \( P \) is atmospheric pressure, \( r_b \) is the boundary layer resistance to diffusion of \( \text{CO}_2 \) and \( r_i \) is the stomatal resistance to diffusion of \( \text{CO}_2 \).

Similarly, for \( ^{13}\text{CO}_2 \),

\[
A = \frac{(p'_a - p'_i)/P}{r'_b + r'_i}, \tag{A1'}
\]

where the primed notation refers to properties related to \( ^{13}\text{C} \).

O'Leary (1981) criticized the use of resistances in computing isotopic fluxes, because the rate of return of material from one pool to a preceding one is not specifically expressed. Instead he treated diffusion as two first-order 'virtual fluxes' in opposite directions. However, the use of resistances, or conductances, to describe diffusion is mathematically
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equivalent, but more succinct, and is also the current notation in other studies of mass and heat transfer from plants. Conductances were used by Farquhar et al. (1982b) in their description of isotopic fractionation in \( C_3 \) species. Resistances and conductances are not, generally, appropriate descriptions of biochemical reactions and are not used in that manner here.

Dividing (A1') by (A1),

\[
\frac{A'}{A} = \frac{P_a - P_i'}{P_a - P} \cdot \frac{r_b + r_l}{r_b' + r_l'}.
\]  

(A2)

It is convenient to introduce the notation ‘quotient’, \( Q \), for the ratio of \( ^{13}\text{C} \) to \( ^{12}\text{C} \) in a particular pool. Thus \( Q_p \) is that of plant material, and under steady conditions is given by

\[
Q_p = \frac{A'}{A}.
\]  

(A3)

Further,

\[
Q_a = \frac{P_a - P_i}{P_a},
\]  

(A4)

\[
Q_i = \frac{P_i}{P_i'},
\]  

(A5)

and

\[
Q_l = \frac{P_i'}{P_i},
\]  

(A6)

where \( p_l \) is the \( p(\text{CO}_2) \) at the leaf surface. Thus (A2) becomes

\[
Q_p = \frac{Q_a P_a - Q_i P_i}{P_a - P_i} \cdot \frac{r_b + r_l}{r_b' + r_l'},
\]

which upon rearrangement yields

\[
\frac{Q_p}{Q_a} = 1 \left[ \frac{P_a - P_i}{P_a} \cdot \frac{r_b + r_l}{r_b' + r_l'} + \frac{Q_i}{Q_p} \cdot \frac{P_i}{P_a} \right].
\]  

(A7)

In turn, following the notation used in equation (3) of the main text

\[
r_b' = r_b/(1 - a_b/1000),
\]  

(A8)

\[
r_l' = r_l/(1 - a/1000),
\]  

(A9)

and, since in this linear chain the resistances are proportional to the drop in \( p(\text{CO}_2) \),

\[
\frac{r_b' + r_l'}{r_b + r_l} = \frac{(P_a - P_l)/(1 - a_b/1000) + (P_i - P_l)/(1 - a/1000)}{P_a - P_i},
\]

Equation (A7) becomes

\[
\frac{Q_p}{Q_a} = 1 \left[ \frac{P_a - P_i}{P_a} \cdot \frac{1}{1 - a_b/1000} + \frac{P_i - P_l}{P_a} \cdot \frac{1}{1 - a/1000} + \frac{P_i}{P_a} \cdot \frac{Q_i}{Q_p} \right].
\]  

(A10)

It now remains to establish a relationship between \( Q_p \) and \( Q \), from an idealized view of the biochemistry.

It is assumed that the \( \text{CO}_2 \) in the intercellular spaces is in equilibrium with that in the cytoplasm of the mesophyll cells. From the standpoint of the mesophyll cells the rate of assimilation of \( \text{CO}_2 \) is given by

\[
A = V_p - L - R_m,
\]  

(A11)

where \( V_p \) is the rate of carboxylation of phosphoenolpyruvate (PEP), \( L \) is the rate of leakage of \( \text{CO}_2/\text{HCO}_3^- \) from the bundle sheath to either the mesophyll cells or the intercellular spaces, and \( R_m \) is the rate of \( ^{12}\text{CO}_2 \) release due to ‘dark respiration’ in the light in the mesophyll cells (and from the epidermal cells into the intercellular spaces). Similarly,

\[
A' = V_p' - L' - R_m'.
\]  

(A11)
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Discrimination occurs during PEP carboxylation and leakage given by

$$V'_p = Q_s (1 - b_4 / 1000) V_p$$  \hspace{1cm} (A12)

and

$$L' = g_s (1 - s / 1000) (Q_s p_s - Q_i p_i)$$,

where

$$Q_s = p' / p_s$$,

and

$$R'_m = Q_p R_m$$,  \hspace{1cm} (A15)

i.e. it is initially assumed that there is no intrinsic discrimination associated with ‘dark’ respiration.

Using (A3) and equations (A12) to (A15), (A11’) is rewritten as

$$Q_p A = Q_s (1 - b_4 / 1000) V_p - g_s (1 - s / 1000) (Q_s p_s - Q_i p_i) - Q_p R_m$$  \hspace{1cm} (A16)

From the standpoint of the bundle sheath cells the carbon fluxes are described by

$$V_c + L = V_p + F + R_s$$

and

$$V'_c + L' = V'_p + F' + R'_s$$,  \hspace{1cm} (A17')

where $$V_c$$ is the rate of RuP₂ carboxylation, $$F$$ is the rate of photorespiration, and $$R_s$$ is the rate of 'dark' respiration in the light in the bundle sheath cells. Discrimination also occurs during RuP₂ carboxylation, according to

$$V'_c = Q_i V_d (1 - b_3 / 1000)$$  \hspace{1cm} (A18)

It is again initially assumed that there is no intrinsic discrimination associated with dark or photorespiration. Thus

$$F' = Q_p F$$  \hspace{1cm} (A19)

and

$$R'_s = Q_p R_s$$  \hspace{1cm} (A20)

Substituting (A12), (A13) and (A18) to (A20) in (A17'), and rearranging,

$$Q_s = \frac{Q_i V_d (1 - b_4 / 1000) + Q_p F + F' + R_s}{V_d (1 - b_3 / 1000) + g_s (1 - s / 1000) p_i}$$  \hspace{1cm} (A21)

After replacing $$(F + R_s)$$ using (A17), the above expression for $$Q_s$$ is substituted into (A16). Upon rearrangement, and using (A11), this yields

$$\frac{Q_i}{Q_p} = \frac{1 - (V_p - L) b_3 + g_s p_i s}{(V_p + g_s p_i) 1000} \left[ 1 - \frac{V_p b_3 + g_s p_i s}{(V_p + g_s p_i) 1000} (1 - b_3 / 1000) \right]$$  \hspace{1cm} (A22)

This is then substituted into (A7) to yield the final solution. However the form differs from that used previously to describe fractionation in C₃ plants, i.e. equation (3) in the main text.

It is instructive to examine (A22) in the case where $$V_p$$ is zero, since this should correspond to a C₃ leaf with liquid phase resistance. Thus,

$$\left( \frac{Q_i}{Q_p} \right)_{V_p=0} = \frac{1 - [(1 - p_i / p_s) b_3 + s p_i / p_s] / 1000}{(1 - s / 1000) (1 - b_3 / 1000)}$$  \hspace{1cm} (A23)
which when substituted into (A7) yields, after rearrangement,

\[
\left( \frac{Q_o}{Q_a} \right)_{v_p=0} = 1 \left[ \frac{P_a-P_l}{P_a} \cdot \frac{1}{1-a/1000} + \frac{P_l-P_i}{P_a} \cdot \frac{1}{1-a/1000} + \frac{P_i-P_s}{P_a} \cdot \frac{1}{1-s/1000} + \frac{P_s}{P_a} \cdot \frac{1}{1-b/1000} \right].
\]  

(A24)

Although related to equation (3), it still differs in form. This is because derivation of (3) by Farquhar et al. (1982b) involved several mathematical approximations of the form

\[
1/(1-x) \approx 1+x,
\]

where \(x\) is small compared to 1. In fact, it is simple to derive an equation for \(C_3\) leaves without these approximations by extending (A7) to

\[
\left( \frac{Q_o}{Q_a} \right)_{C_3} = 1 \left[ \frac{P_a-P_l}{P_a} \cdot \frac{1}{1-a/1000} + \frac{P_l-P_i}{P_a} \cdot \frac{1}{1-a/1000} \right.
\]

\[
+ \frac{P_i-P_s}{P_a} \cdot \frac{1}{1-(1+1+a)/1000} + \frac{P_s}{P_a} \cdot \frac{Q_c}{Q_p},
\]

(A25)

where \(Q_c = p'/p_c\) and \(p_c\) is the \(p(CO_2)\) in equilibrium with the dissolved concentration at the sites of carboxylation. \(Q_c/Q_p\) is then found by summing fluxes:

\[
V_c = A+R_d+F,
\]

(A26)

\[
V_c = A'+R_d+F',
\]

(A26')

where \(R_d\) is the rate of 'dark' respiration. The last equation is rewritten as

\[
Q_c V_c (1-b/1000) = Q_p (A+R_d+F) = Q_p V_c.
\]

Thus

\[
Q_c/Q_p = 1/(1-b/1000)
\]

(A27)

which, when combined with (A25) yields

\[
\left( \frac{Q_o}{Q_a} \right)_{C_3} = 1 \left[ \frac{P_a-P_l}{P_a} \cdot \frac{1}{1-a/1000} + \frac{P_l-P_i}{P_a} \cdot \frac{1}{1-a/1000} + \frac{P_i-P_s}{P_a} \cdot \frac{1}{1-(1+1+a)/1000} + \frac{P_s}{P_a} \right]
\]

\[
\times \frac{Q_c}{Q_p}.
\]

(A28)

Now

\[
\delta - \delta_a = \left( \frac{Q_o}{Q_{PDB}} - 1 \right) \times 1000 - \left( \frac{Q_o}{Q_{PDB}} - 1 \right) \times 1000
\]

\[
= 1000 \left( 1 + \delta_a \frac{1}{1000} \right) \left( \frac{Q_o}{Q_a} - 1 \right).
\]

(A29)

where \(Q_{PDB}\) is the isotopic composition of PDB. It emerges that equation (3) from the main text is a good approximation to this solution for leaves of \(C_3\) species [equation (A28) substituted into (A29)], provided the isotopic composition of the air is similar to that of PDB. As numerical examples, with boundary layer and liquid phase resistances of zero \((p_a = p_l = p_i, a = 4.4\) and \(b_3 = 27\), the maximum error is \(0.13\%\) with \(\delta_a = 0\), and \(0.21\%\) with \(\delta_a = -7.8\).

Returning to the \(C_4\) calculation, equation (A22) can be approximated in many forms but from the above it is clear that the appropriate one is

\[
Q_o/Q_p \approx \left[ 1 - \frac{b_4 V_c + b_3 g_s p_c - sL}{(V_c + g_s) 1000} \right]
\]

\[
= \left[ 1 - \frac{b_4 V_c + b_1 Lp_d (p_c - p_i) - sL}{1000 (V_c + Lp_d (p_c - p_i))} \right]
\]

(A30)
If there is intrinsic discrimination associated with 'dark' and photorespiration, of $e$ and $f$ %o, respectively, equations (A15), (A19) and (A20) are replaced by

$$R'_m = Q_p (1-e/1000)R_m,$$  \hspace{1cm} (A31)

and

$$F' = Q_p (1-f/1000)F,$$  \hspace{1cm} (A32)

while, in equation (A30), $b_4$ is replaced by $(b_4-eR_m/V_p)$ and $b_3$ by $(b_3-[e(R_m+R_d)+fF]/V_c)$.

If the activity of carbonic anhydrase is insufficient to ensure equilibration of $CO_2$ and $HCO_3^-$ in the mesophyll cytoplasm, (A1) is replaced by

$$A+R_m+L = V_h - V_d = V_p,$$  \hspace{1cm} (A34)

and (A1') by

$$A'+R'_m+L' = V'_h - V'_d = V'_p,$$  \hspace{1cm} (A34')

where

$$V'_h = (1-(1+1+x)/1000)Q_c V_h,$$  \hspace{1cm} (A35)

$$V'_d = (1-(x+9.0)/1000)Q_b V_d,$$  \hspace{1cm} (A36)

$1\cdot1\%o$ is the fractionation associated with solution of $CO_2$, $x$ is the fractionation associated with hydration of $CO_2$, and $Q_b$ is the isotopic ratio of bicarbonate in the mesophyll (see Appendix 3). Then (A12) is replaced by

$$V'_p = (1-2\cdot2/1000)Q_b V_p.$$  \hspace{1cm} (A37)

These equations yield

$$Q_b = \frac{(1-(1+1+x)/1000)V_c Q_m}{(1-2\cdot2/1000)V'_p+(1-(x+9.0)/1000)V'_d}. \hspace{1cm} (A38)$$

Using the relation

$$\frac{(1-2\cdot2/1000)(1-(1+1+x)/1000)}{1-(x+9.0)/1000} = 1-b_4/1000,$$  \hspace{1cm} (A39)

it may be shown that, to a good approximation, the earlier results hold with $b_4$ replaced by $[b_4(1-V_p/V_h)+(1+1+x)V'_p/V_h]$.

Appendix 2.

The Diffusion of Inorganic Carbon and Oxygen from the Bundle Sheath Cells to the Mesophyll Cells

The rate of diffusion, $J$ (mol m$^{-2}$ s$^{-1}$), of a substance through a water path of length $l$ (m) is given by

$$J = D \rho \Delta c/l,$$  \hspace{1cm} (B1)

where $D$ (m$^2$ s$^{-1}$) is the diffusivity of that substance in water, $\rho$ (mol m$^{-3}$) is the molar density of the solution and $\Delta c$ (dimensionless) is the difference in concentration (mole fraction) of the substance across the path. In turn, for dissolved gases, the concentration, $c$, may be related to the partial pressure, $p$, which would exist in equilibrium if a gaseous phase were present:

$$c = Hp,$$  \hspace{1cm} (B2)

where $H$ is the Henry coefficient for that gas.

For $CO_2$, the Henry coefficient is $6.02 \times 10^{-4}$ bar$^{-1}$ at 25°C (Cox and Head 1962). Thus the leakage of $CO_2$, $L_c$, from the bundle sheath cells is given by

$$L_c = g_c (p_s - p_m).$$  \hspace{1cm} (B3)
where the conductance to leakage of CO\textsubscript{2} alone is given by
\[
g_{sc} = \rho D_c H_c / l_c ,
\]  
and the subscript \( c \) refers to CO\textsubscript{2}. The effective length, \( l_c \), takes into account the various possible paths via plasmodesmata and the cell wall.

Consider a simplified system, with a uniform pH, where interconversion of CO\textsubscript{2} and HCO\textsubscript{3}\textsuperscript{−} takes place only inside the bundle sheath and mesophyll cells, and not in the pathways of symplastic transport.

The leakage of bicarbonate, \( L_b \), is given by
\[
L_b = g_{sb}(b_s-b_m) = \rho D_b (b_s-b_m) / l_b ,
\]  
where \( b \) refers to bicarbonate, and \( g_{sb} \) is the conductance to leakage of bicarbonate. Combining equations (B3) to (B5), the total leakage of inorganic carbon is given by
\[
L = g_s(p_s-p_m) ,
\]
where
\[
g_s = g_{sc} \left( 1 + D_b (b_s-b_m) / D_c (c_s-c_m) / l_b \right) .
\]
The diffusivity of HCO\textsubscript{3}\textsuperscript{−} in water, \( 1.09 \times 10^{-9} \text{ m}^2 \text{ s}^{-1} \) at 25°C, is 0.56 times that of CO\textsubscript{2}, \( 1.94 \times 10^{-9} \text{ m}^2 \text{ s}^{-1} \) (Kigoshi and Hashitani 1963). Equation (B7) becomes
\[
g_s = g_{sc} \left( 1 + 0.56 (b_s-b_m) / (c_s-c_m) / l_b \right) .
\]

If the CO\textsubscript{2} and HCO\textsubscript{3}\textsuperscript{−} are in equilibrium,
\[
b_s-b_m = K(c_s-c_m) / [H^+] ,
\]
where \( K \) is the dissociation constant for HCO\textsubscript{3}\textsuperscript{−}, equal to \( 10^{-6.35} \) at 25°C (Edsall 1969); \( K \) is greater in the presence of salts. For many other parameters the salt effects are unknown, and, consistently, they are all ignored here.

If CO\textsubscript{2} and HCO\textsubscript{3}\textsuperscript{−} are not in equilibrium in the bundle sheath cells because of insufficient amounts of carbonic anhydrase, the concentration of HCO\textsubscript{3}\textsuperscript{−} will only be a fraction, \( \alpha_s \), say, of its equilibrium value. An upper bound on \( \alpha_s \) is 1. A lower bound may be determined by assuming that the rates of interconversion of CO\textsubscript{2} and HCO\textsubscript{3}\textsuperscript{−} are those occurring in pure water, but since the latter are so low this bound is close to zero.

The rate of production of HCO\textsubscript{3}\textsuperscript{−} is matched by the sum of the rates of the reverse reaction and the leakage of HCO\textsubscript{3}\textsuperscript{−} from the bundle sheath cells. Thus
\[
k_c p_s c_s = k_b p_s b_s + g_{sb}(b_s-b_m) ,
\]
where \( p_s \) (mol H\textsubscript{2}O per m\textsuperscript{2} leaf surface) is the areal density of bundle sheath cell water and \( k_b \) and \( k_c \) are effective rate constants (s\textsuperscript{-1}). In water
\[
k_b = 2 \times 10^{-4} + 10 \times 5 \times 10^{[\text{H}^+]}
\]
and
\[
k_c = 8 \times 5 \times 10^{-11} / [\text{H}^+] + 3.7 \times 10^{-2} ,
\]
with [\text{H}^+] (mol l\textsuperscript{-1}) being the concentration of H\textsuperscript{+}. Dr I. R. Cowan derived equations (B11) and (B12) from others of Miller and Colman (1980). As the activity of carbonic anhydrase increases, \( k_b \) and \( k_c \) increase, but in constant ratio.

From equation (B10)
\[
b_s = k_c p_s c_s + b_m g_{sb} / p_s
\]
\[
and
\]
\[
b_s-b_m = k_c p_s c_s - k_b p_s b_m / k_b + g_{sb} / p_s .
\]

Hence,
Assuming that CO₂ and HCO₃⁻ are in equilibrium in the mesophyll cells,

\[ k_b b_m = k_c c_m \]

and so, substituting this expression in equation (B13),

\[ b_s - b_m = \frac{k_c}{k_b + g_{sb}/\rho_s} (c_s - c_m) . \]

Incorporating this in equation (B8) and using

\[ k_c/k_b = K/[H^+] , \]

where

\[ g_s = g_{sc} \left( 1 + 0.56\alpha_s K_L/[H^+] \right) , \]

\[ \alpha_s = \frac{1}{1 + g_{sb}/(k_b \rho_s)} . \]

In the presence of sufficient carbonic anhydrase, \( \alpha_s \) approaches 1, as indicated by equation (B16).

The extent to which HCO₃⁻ equilibrates with CO₂ and diffuses along the same path is defined as \( \alpha_s \), i.e.

\[ \alpha = \alpha_s l_s/l_b . \]

Since the effective pathlength for bicarbonate diffusion is longer than that for CO₂ and since \( \alpha_s \) is less than unity,

\[ 0 < \alpha < 1 . \]

Equation (B15) may be rewritten as

\[ g_s = g_{sc}(1 + 0.56 \alpha K/[H^+]) . \]

The pH in the cytoplasm is approximately 7.4 (Smith and Raven 1979) and so equation (B19) may be simplified to

\[ g_s = g_{sc}(1 + 6.3\alpha) . \]

The diffusion of oxygen from the bundle sheath, \( J_o \), is given by

\[ J_o = \frac{D_o H_o l_s}{D_c H_c l_b} g_{oc}(p_{oc}-p_{om}) , \]

where the subscript, o, refers to oxygen. The Henry coefficient for O₂ is \( 2.27 \times 10^{-5} \text{bar}^{-1} \) at 25°C (Battino and Clever 1966), which is 0.038 times that for CO₂ at 25°C; and the diffusivity of O₂ in water is \( 2.43 \times 10^{-9} \text{m}^2 \text{s}^{-1} \) at 25°C (Andrussow 1969), which is 1.26 times that of CO₂, \( 1.93 \times 10^{-9} \text{m}^2 \text{s}^{-1} \) at 25°C (Andrussow 1969).

If it is assumed that the effective path lengths for dissolved CO₂ and O₂ are identical,

\[ J_o = g_{oc}(p_{oc}-p_{om})/21.1 \]

and

\[ L/J_o = 21.1(1+6.3\alpha)(p_s-p_m)/(p_{oc}-p_{om}) . \]

Using equation (A28), the leakiness, \( \phi \), is given by

\[ \phi = \frac{L}{V_p} = \frac{L}{A+R_m+L} , \]

where \( R_m \) is the dark respiration rate in the mesophyll cells and is small compared to the net assimilation rate, \( A \).

It is algebraically convenient to define \( \beta \) using

\[ J_o = \beta(A+R_m) . \]
Using equations (B23) and (B25) the expression for $\phi$ may be rewritten as

$$\phi = \frac{21 \cdot 3\beta(1+6 \cdot 3\alpha)(p_t-p_m)}{p_o-p_m} + 21 \cdot 3\beta(1+6 \cdot 3\alpha)(p_t-p_m).$$

(B26)

**Appendix 3. Fractionation Associated with Leakage**

In Appendix 2 it was shown that the leakage of $^{12}\text{CO}_2$ and $^{13}\text{CO}_2$ is given by

$$L = \frac{g_{sc}}{D_b} \left( 1 + \frac{D_b}{D_c} \frac{k_c}{(k_b+g_{sc}/\rho_s)l_b} \right) (p_s-p_m).$$

(C1)

It is known that the equilibrium constant, $K'$, between $^{13}\text{CO}_2$ and dissolved $^{12}\text{CO}_2$ differs from $K$, that for $^{12}\text{CO}_3$ and $^{12}\text{CO}_2$, by 9.0%, causing a slight enrichment of $^{13}\text{CO}_2$. However the cause of this is unknown. Thus

$$k_c' = k_c(1-x/1000)$$

and

$$k_b' = k_b[1-(a+g)/1000],$$

where $x$ is, at this stage, unknown, but presumably positive, and the prime notation refers to $^{13}$C. The fractionation associated with leakage caused by this effect may be calculated from the above equations, assuming $l_c/l_b = 1$. If there are isotopic effects on the diffusivities of $\text{CO}_2$ and $\text{HCO}_3$ in water, with associated fractionations of $a_1$ and $a_b$ % respectively, then since at $25^\circ\text{C} H_c' = H_c(1-1.1/1000),$

$$L' = \frac{g_{sc}}{D_b} \left( 1 + \frac{D_b}{D_c} \frac{k_c}{(k_b+g_{sc}/\rho_s)l_b} \right) \left( 1 + \frac{0.56(1-(a_{ib}-a)/1000)k_c(1-x/1000)l_c/l_b}{k_b(1-(a+g)/1000)+g_{sc}(1-a_{ib}/1000)l_b} \right) (p_s'-p_m).$$

(C1')

$$= g_{sc}(1+6 \cdot 3\alpha)(1-s/1000)(p_s'-p_m),$$

where

$$s = \frac{6 \cdot 3\alpha}{1+6 \cdot 3\alpha} \cdot \frac{(x-a)g_{sc}((\rho_s/l_b)+(a_{ib}-a)-9 \cdot 0)}{1+g_{sc}/(\rho_s/k_b)} + 1 + a_1,$$

and $\alpha$ is defined by equation (B17). The factor 6.3 is the approximate numerical value of $KD_b/([H^+]D_c)$ at pH 7.4.

From equation (B15)

$$g_s = g_{sc}(1+6 \cdot 3\alpha),$$

and so equation (C4) provides an expression for the fractionation ($s/100$) associated with leakage. Since $0<\alpha<1$, $s$ cannot be more negative than $-6.7$ (again assuming $x>0$), and this would occur only in the presence of substantial carbonic anhydrase activity in the bundle sheath cells, and if the effective pathlength for $\text{HCO}_3$, $l_b$, did not exceed that for $\text{CO}_2$, $l_c$.

Note that, in Appendix 2 and implicitly here, it was assumed that $\text{HCO}_3$ is in equilibrium with $\text{CO}_2$ in the mesophyll cytoplasm.

*Note added in proof:* R. J. Francey (unpublished) found that $\delta_{\text{CO}_2}$ in southern hemisphere maritime air had a mean value of $-7.6\%$ with a seasonal variation of less than $0.05\%$ in 1982.

M. H. O’Leary (unpublished) has determined $a_1$ to be 0.7 at 25°C.

Manuscript received 11 August 1982, accepted 2 February 1983