Plant Physiol. (1995) 108: 173–181

Measurement of the Leakage of CO_2 from Bundle-Sheath Cells of Leaves during C_4 Photosynthesis

Marshall D. Hatch*, Anthony Agostino, and Colin L. D. Jenkins

Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, GPO Box 1600, Canberra, Australian Capital Territory, 2601 Australia

During C_4 photosynthesis, CO_2 is released in bundle-sheath cells by decarboxylation of C_4 acids and then refixed via ribulose-1,5-bisphosphate carboxylase. In this study we examined the efficiency of this process by determining the proportion of the released CO_2 that diffuses back to mesophyll cells instead of being refixed. This leak of CO_2 was assessed by determining the amount of $^{14}CO_2$ released from leaves during a chase in high [$^{12}CO_2$] following a 70-s pulse in $^{14}CO_2$. A computer-based analysis of the time-course curve for $^{14}CO_2$ release indicated a first-order process and provided an estimate of the initial velocity of $^{14}CO_2$ release from leaves. From this value and the net rate of photosynthesis determined from the $^{14}CO_2$ fixed in the pulse, the CO_2 leak rate from bundle-sheath cells (expressed as a percentage of the rate of CO_2 production from C_4 acids) could be deduced. For nine species of Gramineae representing the different subgroups of C_4 plants and two NAD-malic enzyme-type dicotyledonous species, the CO_2 leak ranged between 8 and 14%. However, very high CO_2 leak rates (averaging about 27%) were recorded for two NADP-malic enzyme-type dicotyledonous species of *Flaveria*. The results are discussed in terms of the efficiency of C_4 photosynthesis and observed quantum yields.

The function of the unique reactions of C_4 photosynthesis is to concentrate CO_2 in bundle-sheath cells for assimilation via Rubisco (Fig. 1; Hatch, 1987). In this way photorespiration and its adverse effects on photosynthetic efficiency are largely abolished. There is an energy cost for this concentrating process that is equivalent to the two ATP molecules consumed by pyruvate Pi dikinase in the course of generating one molecule of the primary CO_2-acceptor PEP (Hatch, 1987). As a result, any CO_2 that leaks from bundle-sheath cells, rather than being refixed via Rubisco, will reduce the efficiency of C_4 photosynthesis. The effects of this CO_2 leakage on the quantum requirements for C_4 photosynthesis were analyzed previously (Farquhar, 1983; Furbank et al., 1990). In the latter study we showed that, if only 50% of the CO_2 generated in bundle-sheath cells is usefully refixed, then the calculated quantum yield reduces from the highest recorded value for C_4 plants to a value close to that observed for C_3 plants (Furbank et al., 1990).

Some leakage of CO_2 from bundle-sheath cells would be unavoidable at least to the extent that CO_2 must diffuse out through the numerous plasmodesmata present in the mesophyll-bundle-sheath cell wall interface (Hatch, 1987). Normally, CO_2 diffuses rapidly through the cell membrane and wall, but in certain C_4 plants the presence of a suberin layer in the cell wall is believed to limit this diffusion. We have modeled these relationships (Jenkins et al., 1989b) and also have measured the permeability coefficient for the transfer and assimilation of CO_2 by bundle-sheath cells (Furbank et al., 1989; Jenkins et al., 1989a). Compared to C_3 photosynthetic cells, a much higher resistance to the diffusion of CO_2 to the site of assimilation in C_4 bundle-sheath cells was found for both an isolated cell system and intact leaves.

There have been two approaches to assessing the extent of the leakage of CO_2 from bundle-sheath cells in C_4 plants. One procedure is based on measurement of carbon-isotope discrimination. Earlier estimates using this procedure (Farquhar, 1983) gave values for leakiness (the fraction of CO_2 generated by C_4 acid decarboxylation that subsequently leaks from bundle-sheath cells) of up to 0.5. More recent estimates were in the range of 0.3 to 0.47, but lower values in the range of 0.2 to 0.3 were obtained using measurements of short-term isotope discrimination (Henderson et al., 1992). A general estimate of the fractional CO_2 leak from bundle-sheath cells of 0.14 was derived from a model of the inorganic carbon status of C_4 bundle-sheath cells using "best estimate" values of various parameters, including measurements of the CO_2 permeability across the mesophyll-bundle-sheath interface (Jenkins et al., 1989b). This result was supported by other studies using a similar approach (Brown and Byrd, 1993). In the present paper we describe a simple radiotracer pulse-chase procedure for estimating the leakage of CO_2 from bundle-sheath cells of C_4 leaves under steady-state conditions for photosynthesis.

MATERIALS AND METHODS

Biochemicals and enzymes were obtained from Sigma or from Boehringer-Mannheim. High specific radioactivity $Ba^{14}CO_3$ (about 55 Ci mol^{-1}), used for generating $^{14}CO_2$ gas, was obtained from Amersham. Hyamine (methylbenzethonium hydroxide) was obtained from Sigma.

Plant Material

Plants were grown in soil in a naturally illuminated glasshouse maintained at day/night temperatures of 28/

* Corresponding author; e-mail hal.hatch@pican.pi.csiro.au; fax 61–6–2465000.

Abbreviations; C_a, external CO_2 concentration; C_i, substomatal CO_2 concentration; NAD-ME type, NAD-malic enzyme type; NADP-ME type, NADP-malic enzyme type.

20°C between September and April and 25/15°C for the remaining winter period. Plants received supplementary light of about 200 μmol m^{-2} s^{-1} PPFD for the winter period. Except where indicated otherwise, top, fully expanded leaves were used from young plants (2–5 weeks old) in a stage of rapid, vegetative growth. For maize (*Zea mays* L.), the third leaf was fully expanded by about 13 d after germination in summer and by about 21 d during the mid-winter period.

Radiotracer Pulse-Chase Procedure for Measuring CO_2 Leakage

Leaves of grass species were detached from plants during the mid-morning period and then recut under water to obtain a midsection approximately 14 cm long. Between two and four leaves, depending on their width (between 1 and 2 mg of total Chl), were then placed in a Perspex photosynthesis chamber, with the basal end in water and the top protruding through a split lid lined with a foam strip to form a seal. Whole leaves of dicotyledonous plants were placed in the chamber with the petiole protruding through the foam sealing strips and the cut end wrapped in tissue paper saturated with water. The chamber (3-L volume with two high-speed fans to rapidly circulate the air) was maintained at 25°C and was illuminated with a 400-W Phillips mercury vapor lamp to give 800 μmol m^{-2} s^{-1} PPFD at the leaf's surface. It was gassed with about 5 L min^{-1} humidified air supplemented with air containing 2000 μL L^{-1} CO_2, so that the final steady chamber concentration was maintained at about 400 μL L^{-1} CO_2. This was achieved by measuring the CO_2 concentrations of the chamber gas (50 mL sampled with a syringe and measured with an IRGA [ADC, LCA-2, Hoddeston, Herts, UK]) and then adjusting the input flow of the CO_2 mixture. The exact chamber CO_2 concentration was measured just prior to adding $^{14}CO_2$ so that a precise specific radioactivity could be calculated. This CO_2 concentration varied between 390 and 410 μL L^{-1} in different experiments. The leaves were maintained under the above conditions for about 30 min to establish a steady rate of photosynthesis, and then the $^{14}CO_2$ pulse commenced by removing the air input and immediately injecting about 0.2 mCi (approximately 4 μmol) of $^{14}CO_2$ gas. At about 10 and 60 s after $^{14}CO_2$ was added, gas samples were removed from the chamber to determine the ^{14}C content of the gas phase. The $^{14}CO_2$ in these samples was trapped by injecting it through Subaseal stoppers into flasks containing 30% (v/v) hyamine in methanol, and after shaking for 3 h, samples were counted by a liquid scintillation procedure. From these values the specific radioactivity of the chamber $^{14}CO_2$ could be determined.

After 70 s the lid of the pulse chamber (together with the leaves) was quickly transferred to the chase chamber, which had been previously flushed with an air mixture to give a final CO_2 concentration of about 1000 μL L^{-1} (or as otherwise specified). The transfer took about 2 s. The 3-L chase chamber was illuminated in exactly the same way as the pulse chamber, with the same temperature and with the air circulated by high-speed fans.

The kinetics of $^{14}CO_2$ release from the leaves was determined by taking 10-mL gas samples from the chase chamber at about 2, 4, 7, 10, 15, 20, and 30 s after the transfer to the chase chamber was completed. This was performed with a battery of syringes set up in a frame, with needles protruding into the chamber through serum stoppers placed in the Perspex side wall. The precise time that each syringe was operated was recorded by a computerized, stopwatch program. Later, the $^{14}CO_2$ in these samples was trapped in hyamine and counted, as described above.

After the 30-s gas sample was taken, the leaves were immediately removed from the chase chamber and killed in 30 mL of boiling 80% (v/v) ethanol in water. Then, the leaves were extracted by blending in the killing mixture in a Sorvall Omnimixer (1 min at full speed). After the sample was centrifuged, the supernatant fluid was removed, and the residual, insoluble material was extracted successively with 12 mL of 80% (v/v) ethanol, 12 mL of 50% (v/v) ethanol, and 15 mL of water. After each extraction the clear extract obtained by centrifugation was pooled with the original 80% ethanol extract. The total fixed ^{14}C was determined by counting samples of the combined, soluble extracts and samples of the residual, insoluble material (for ^{14}C in starch, see Hatch, 1979). Chl was extracted from the soluble fraction with chloroform, and samples were used to determine the Chl content measured as pheophytin (Vernon, 1960). For the experiments in which the distribution of fixed radioactivity among different compounds was determined, including the degradation of malate and aspartate to determine the loss of ^{14}C from the C-4 carboxyl during the chase, analyses were carried out as previously described (Hatch, 1979).

Determination of Rates of Photosynthesis and CO_2 Leakage

The photosynthesis rate of leaves was determined from the specific radioactivity of the $^{14}CO_2$ in the pulse chamber and the total ^{14}C fixed by leaves during the 70-s pulse. The CO_2 leak rate was determined from the initial rate of $^{14}CO_2$ release from leaves computed from the curve for $^{14}CO_2$ release as follows. Data for $^{14}CO_2$ accumulation in the chase chamber (y) and time for chase (t) were fitted to the equation:

$$y = A - Be^{-kt} \quad (1)$$

where A, B, and k are constants determined by an iterative, nonlinear curve-fitting procedure (simplex method) on a computer (Fig. 2). This equation describes the accumulation of the product (released $^{14}CO_2$) from a first-order process with a rate constant k. The parameter A corresponds to the asymptote value of $^{14}CO_2$ accumulated in the chase chamber (i.e. from about 2 s after the start of the chase), and B corresponds to the asymptote value of total $^{14}CO_2$ released in the chase from time zero. Thus, the value of B is greater than A, and from the difference the negative intercept on the y axis at zero time can be determined. The initial rate of $^{14}CO_2$ release from the leaves is given simply by $B \times k$.

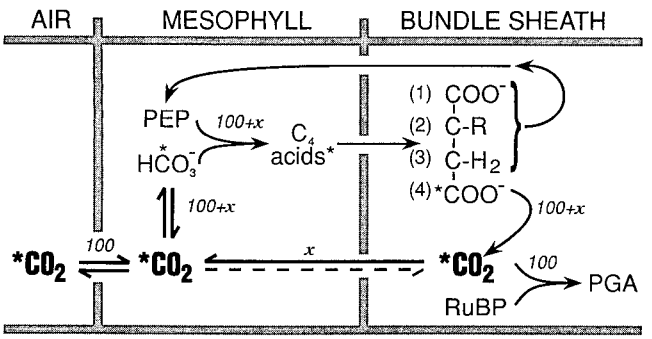

Figure 1. A simple scheme for C_4 photosynthesis showing the key fluxes of inorganic carbon. Taking the net assimilation of carbon as 100 and the leakage of CO_2 from bundle-sheath cells as x, then the C_4 cycle will operate at a rate of 100 + x. PGA, 3-Phosphoglycerate; RuBP, ribulose 1,5-bisphosphate.

Measurement of Leaf CO_2-Saturation Curves and Substomatal CO_2

CO_2-response curves for leaf photosynthesis were determined by measuring CO_2 exchange with an IRGA and a Parkinson leaf chamber (LCA-2 and PLC-B, ADC). These determinations were conducted under the specific conditions of our $^{14}CO_2$ pulse-chase experiments (800 μmol m^{-2} s^{-1} PPFD, 25°C, and leaves of the same age). The C_i was determined also under the same conditions, using the procedure described by von Caemmerer and Farquhar (1981). These values were used to develop a correction to account for $^{14}CO_2$ released from bundle-sheath cells but refixed in mesophyll cells (see "Results and Discussion").

Kinetics of Change in Stomatal Conductance

Detached leaves, with the basal end in water, were allowed to establish a steady rate of photosynthesis in the leaf chamber of an IRGA (see above) under conditions similar to those used for the $^{14}CO_2$ pulse-chase studies (about 850 μmol m^{-2} s^{-1} PPFD, 25°C, but RH was about 50%). Dry air was supplied, and the steady RH in the chamber of about 50% (measured by the chamber humidity sensor and traced on a chart recorder) reflected leaf transpiration. Changes in stomatal conductance monitored as RH were measured following rapid changes in the $[CO_2]$ in the IRGA chamber from about 350 to 900 μL L^{-1}. The response time with this system was less than 2 s. From this and measurements of air and leaf temperatures, stomatal conductance could be calculated (von Caemmerer and Farquhar, 1981).

RESULTS AND DISCUSSION

Measurement of CO_2 Leakage from Bundle-Sheath Cells

Basic Methodology

CO_2 leakage from bundle-sheath cells was determined by a procedure involving a radiotracer pulse chase. As described in detail in "Materials and Methods," leaves were allowed to assimilate $^{14}CO_2$ under steady-state conditions for photosynthesis for a period sufficient to permit the C-4 carboxyl of C_4 acids to saturate with respect to ^{14}C (Fig. 1). The data from earlier studies indicate saturation of the C-4 carboxyl in 30 to 60 s for different species under these conditions (Hatch, 1971, 1979; Furbank and Hatch, 1987).

We chose 70 s as a routine time for this $^{14}CO_2$ pulse. At 70 s leaves were transferred to a chase chamber containing unlabeled CO_2 but with the same temperature and light, and the release of $^{14}CO_2$ from leaves into this chamber was determined from gas samples taken at intervals during the following 30 s. In a large number of experiments conducted with different species under our particular conditions, $^{14}CO_2$ release approached a plateau value after a chase period of about 30 to 40 s. This is very similar to the time required in previous experiments to chase ^{14}C from the C-4 carboxyl of C_4 acids (Hatch, 1971, 1979). Notably, since C_4 acids are the initial products of $^{14}CO_2$ fixation, the decline of C-4 radioactivity in a chase should be first order. This released $^{14}CO_2$ was taken as a measure of the $^{14}CO_2$ that had effluxed from bundle-sheath cells to mesophyll (instead of being refixed via Rubisco) and then diffused from the leaf into the chase chamber gas phase (Fig. 1). A computer program was used that fitted these data to a "best fit" first-order curve (Fig. 2, see "Materials and Methods" for details), and from this the initial velocity of $^{14}CO_2$ release in the chase could be calculated together with other parameters (including the rate constant for the $^{14}CO_2$-release process). Of about 65 experiments with 14 different C_4 species, in only two cases (both with *Amaranthus edulis* leaves) were the data rejected by the program as not being a good, first-order fit. As already noted, the C-4 carboxyl of C_4 acids, which is the source of this $^{14}CO_2$, was saturated with ^{14}C at the start of the chase. Therefore, it initially will have the same specific radioactivity as the $^{14}CO_2$ provided

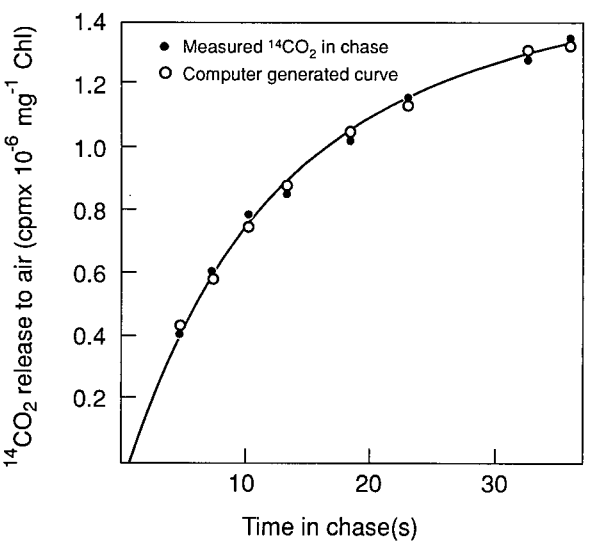

Figure 2. Typical curve for $^{14}CO_2$ release from C_4 leaves during a chase in $^{12}CO_2$ following a pulse in $^{14}CO_2$. Observed values and those for a computer-generated best-fit first-order curve are shown. The data are for *Z. mays* leaves and the experiment was conducted under our standard conditions (see "Materials and Methods").

in the pulse stage so that the initial rate of $^{14}CO_2$ release can be compared directly with the net photosynthesis rate for the same leaves determined directly from the $^{14}CO_2$ fixed in the 70-s pulse period. Clearly, this procedure would provide a simple and direct measure of the total leakage of CO_2 from bundle-sheath cells provided that there was no refixation via PEP carboxylase (Fig. 1) or that a correction could be applied for any refixation that occurred.

Refixation of Leaked CO_2 in Mesophyll Cells

We reasoned that, if there is significant refixation of leaked $^{14}CO_2$ with atmospheric levels of CO_2 in the chase, then this should be eliminated by increasing the chase-CO_2 concentration to above this level. Under these circumstances the higher chase-CO_2 concentration should, in the short term, give a near instantaneous and proportional increase in the mesophyll-CO_2 concentration. This should, in turn, increase the rate of exchange of mesophyll-cell CO_2 with air without any increase in the already saturated PEP carboxylase reaction (Fig. 1). This conclusion depends on two assumptions: first, that C_4 photosynthesis is already saturated at 400 μL L^{-1} CO_2 and, second, that there would be no significant change in stomatal conductance in the first 30 s after the transfer from 400 μL L^{-1} CO_2 to a higher concentration.

In support of the first assumption, we showed that photosynthesis in six C_4 species that we examined was saturated at between 310 and 350 μL L^{-1} CO_2 under the conditions used for our pulse-chase experiments. With regard to the second point, there is evidence that this assumption is valid for leaves of C_3 plants and for the C_4 species, sugarcane (*Saccharum officinarum*) (Meidner and Mansfield, 1968; Grantz and Zeiger, 1986). To confirm this for the species used in the present study, we monitored stomatal conductance by measuring the change in humidity following rapid changes in the CO_2 concentration in air supplied to leaves photosynthesizing at a steady rate in an IRGA chamber (see "Materials and Methods"). For the eight C_4 species we examined, there was no detectable change in stomatal conductance during the first 30 s after the CO_2 concentration was rapidly altered in the range from about 350 to 900 μL L^{-1}. The onset of change was usually detected within 1 min, with the new steady state being approached about 5 min thereafter. The curve for changing stomatal conductance was sigmoidal in shape, and the average decrease in conductance for the C_4 grasses was about 38%. For the C_3 plant wheat, conductance did not start to decline for at least 2 min after the CO_2 concentration was increased. The change took about 8 min to complete, followed by some oscillatory behavior.

If the above arguments and propositions are accepted, it is possible to generate a theoretical curve, demonstrating the plateauing of $^{14}CO_2$ release from leaves as the chase $[CO_2]$ is increased. From the C_i and photosynthesis rates measured for several C_4 species under the conditions of our experiments (see below), we calculated that this plateauing should be clearly apparent at a C_a of about 1500 μL L^{-1} and above (Fig. 3). For the theoretical curve shown, the proportion of leaked CO_2 effluxing to the air increases from about

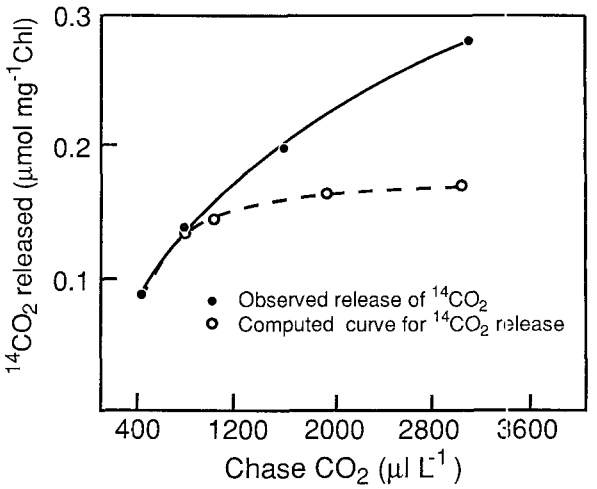

Figure 3. Effect of increasing the CO_2 concentration used in the chase chamber on the observed $^{14}CO_2$ release from leaves. Total $^{14}CO_2$ release was computed from the observed first-order curve for $^{14}CO_2$ release obtained with the different chase-CO_2 concentrations. The basis for calculating the expected theoretical response to increasing the chase CO_2 is described in the text.

49% at 410 μL L^{-1} C_a, to 82% at 1200 μL L^{-1} CO_2, and to 94% at 3000 μL L^{-1} CO_2. However, we observed a somewhat larger increase in both the initial rate and the total amount of $^{14}CO_2$ released as the chase-CO_2 concentration was increased to about 3000 μL L^{-1} (Fig. 3).

At this point it was realized that higher C_a values in the chase are likely to result in an overestimation of the leakage of $^{14}CO_2$ from bundle-sheath cells because of the increased flux of unlabeled CO_2 into bundle-sheath cells (Fig. 1). For instance, it can be calculated that when the C_a is increased from 400 to 3000 μL L^{-1} under our experimental conditions the steady-state mesophyll cell $[CO_2]$ will transiently increase about 13-fold to 2810 μL L^{-1}, assuming that there is no adjustment of stomatal conductance in the short term (see above). The flux of unlabeled CO_2 into bundle-sheath cells would increase by a similar factor causing an increase in the steady-state pool of CO_2 in bundle-sheath cells and a significant dilution of the $^{14}CO_2$ generated in these cells. Under these circumstances reassimilation of released $^{14}CO_2$ via Rubisco will be decreased as the specific radioactivity of the bundle-sheath CO_2 pool declines. This decrease will be most evident in the likely event that Rubisco in bundle-sheath cells is already close to being saturated by the bundle-sheath cell CO_2 concentration prevailing with the lower levels of C_a (Jenkins et al., 1989b). The reduction in $^{14}CO_2$ refixation will be matched by an increased leak of $^{14}CO_2$ to mesophyll cells, causing an overestimation of the true leak of CO_2 occurring under the conditions of the pulse treatment.

Conditions and Corrections

As a result of the above considerations, we adopted the following set of experimental conditions to measure the leakage of CO_2 from bundle-sheath cells as a compromise. After a pulse in about 400 μL L^{-1} CO_2, the release of $^{14}CO_2$

was measured at a chase-CO_2 concentration of about 1000 μL L^{-1}. At this concentration the overestimation effect discussed above is minimal (Fig. 3), but refixation of leaked $^{14}CO_2$ via PEP carboxylase in mesophyll cells remains significant. However, the following considerations indicate that under these conditions only a small part of the $^{14}CO_2$ released into mesophyll cells will be refixed and that a precise correction factor for this refixation can be calculated.

For this calculation we modeled the system described in Figure 1 using the following values: an initial C_a in the pulse of 410 μL L^{-1} and a net photosynthesis rate (i.e. CO_2 fixed by Rubisco) of 4.6 μmol min^{-1} mg^{-1} Chl (average values for a large number of pulse-chase experiments conducted with fully expanded leaves illuminated at 800 μmol m^{-2} s^{-1}, 25°C, and a RH of more than 90%) and a C_i/C_a ratio of 0.52 ± 0.07. This value was obtained by correcting the average of the C_i/C_a values we recorded for seven C_4 species (0.43 ± 0.06) for the effect of the higher humidity prevailing in our pulse-chase experiments using the curve published by Morison and Gifford (1983).

For the purposes of this calculation, we assumed a total CO_2 leakage rate of 15% of net photosynthesis (the median value for a large number of determinations corrected for refixation by PEP carboxylase, see following data). Accordingly, the leakage of CO_2 from bundle-sheath cells to mesophyll cells would be 0.7 μmol min^{-1} mg^{-1} Chl (15% of 4.6), and the rate of C_4 acid synthesis and decarboxylation (C_4 acid cycle) would be 5.3 μmol min^{-1} mg^{-1} Chl. With a C_i/C_a value of 0.52, the C_i would be 214 μL L^{-1} with a C_a of 410 μL L^{-1}. From this it can be deduced that a net flux of 4.6 μmol CO_2 min^{-1} mg^{-1} Chl from air to the mesophyll cells must be sustained by a CO_2 gradient of 196 μL L^{-1} and, by simple proportion, the gross influx and efflux rates of CO_2 between air and the mesophyll cells would be 9.6 and 5.0 μmol min^{-1} mg^{-1} Chl, respectively. Thus, the fraction of $^{14}CO_2$ leaking from bundle-sheath cells to mesophyll cells that actually escapes to the air can be calculated from the relative rates of refixation and efflux (i.e. 5.0/[5.0 + 5.3] = 0.485); that is, slightly less than half of the leaked CO_2 would be released to the air with a C_a value of 410 μL L^{-1}.

However, in our experiments the C_a in the chase phase of the experiment was increased to 1000 μL L^{-1}. If we assume that in the brief period of the chase (30 s) there would be no significant adjustment of stomatal conductance due to this higher CO_2 concentration (see above), then with a C_a of 1000 μL L^{-1} and the same gradient into mesophyll cells, the mesophyll CO_2 would rapidly adjust to 804 μL L^{-1}. Under these conditions CO_2 influx and efflux rates between mesophyll cells and air change to 23.4 and 18.7 μmol min^{-1} mg^{-1} Chl, respectively. With this potential for CO_2 efflux, the fraction of leaked $^{14}CO_2$ escaping to air is increased to 0.78 (18.7/24.0). Thus, the factor for correcting the observed rate of $^{14}CO_2$ leakage to give the total leakage rate would be 1.28 (i.e. 1/0.78).

For this analysis we have taken C_i as a reasonable approximation of the mesophyll cell CO_2 concentration. Of course, the mesophyll cell CO_2 concentration must be less than that in the intercellular air spaces, but the magnitude of this gradient is not known. In this connection it is significant that a high proportion of the mesophyll cell surface is in contact with air spaces, 4 to 5 times the area in contact with bundle-sheath cells (Dengler et al., 1994). von Caemmerer and Evans (1991) have estimated that the CO_2 gradient between the intercellular air spaces and the chloroplasts of C_3 plants may be as much as 75 μL L^{-1}. If the gradient into the mesophyll cytosol of C_4 plants is similar, then the factor for correcting the observed rate of the $^{14}CO_2$ leak would be increased from 1.28 to 1.41. That is, all of the leak-rate values quoted below would be about 10% higher.

It should be noted that the magnitude of this correction factor is only slightly changed (less than 3%) by varying the assumed leak rate in the range from 10 to 20% and is not affected by the value assumed for the photosynthesis rate. Varying C_a in the chase between 950 and 1050 μL L^{-1} (the extremes of the range of C_a values used in our studies) gave less than a 2% change in the correction factor. Variations in the ratio of C_i/C_a between 0.45 and 0.6 (extremes of range observed) compared to the average value we used of 0.52 caused variations in the correction factor of less than 5%. Accordingly, we used the factor of 1.28 times to correct the observed rate of $^{14}CO_2$ production when our standard experimental conditions were used. When variations in critical paramaters were beyond the range of these experiments, the true rate of CO_2 efflux from bundle-sheath cells can be calculated from the equation:

$$L = \frac{L_{ob}\,A\{1 + [C_{i(chase)}/(C_a - C_i)]\}}{[C_{i(chase)}A/(C_a - C_i)] - L_{ob}} \qquad (2)$$

where L and L_{ob} are the true leak rate and the observed leak rate of $^{14}CO_2$, respectively, A is the net rate of CO_2 assimilation (photosynthesis rate) all expressed as cpm min^{-1} mg^{-1} Chl, C_a and C_i are the external and substomatal CO_2 concentrations prevailing in the pulse treatment, respectively, and $C_{i(chase)}$ is the substomatal CO_2 concentration in the chase phase of the experiment. With the value of 0.52 for C_i/C_a (see above), the term $C_{i(chase)}$ can be replaced by $[C_{a(chase)} - 0.48\,C_a]$ and C_i by 0.52 C_a, where $C_{a(chase)}$ is the C_a in the chase.

CO_2 Leakage from *Z. mays* Leaves

Reproducibility

Maize leaves were chosen for detailed studies of the CO_2 leak. To test the reproducibility of the procedure, the leak of CO_2 was determined on the third leaf of plants grown in the glasshouse at different times throughout the year. In seven, separate determinations under our standard conditions (25°C, 800 μmol m^{-2} s^{-1} PPFD, 400 μL L^{-1} CO_2 in the pulse chamber and 1000 μL L^{-1} CO_2 in the chase chamber; see "Materials and Methods" for other details), the leak rate, expressed as a percentage of the rate of CO_2 release in bundle-sheath cells, averaged 14.2 ± 1.4% (Table I). The range of values for the seven, separate experiments was 12.4 to 16.3%. This average value is derived from the leak expressed as a percentage of the net photosynthesis rate, which averaged 16.5 ± 2% (see footnotes for Table I).

Table I. *Averages for $^{14}CO_2$ leak kinetics and photosynthesis rate for seven, separate determinations on the third leaf of maize*

Averages with SD for seven, separate experiments. Observed ranges of values are given in parentheses. $^{14}CO_2$ pulse-chase experiments were conducted under standard conditions (800 μmol m^{-2} s^{-1} PPFD, 25°C, 1000 μL L^{-1} CO_2 in chase, see "Materials and Methods" and text for other details). Photosynthesis rate was determined from $^{14}CO_2$ fixed in the pulse.

Photosynthesis Rate	Total $^{14}CO_2$ Released from Leaves[a]	Rate Constant[a]	Rate of CO_2 Leak from Bundle-Sheath Cells	
μmol $min^{-1}mg^{-1}$ Chl	*μmol mg^{-1} Chl*	*s^{-1}*	*% of net photosynthesis rate[b]*	*% of rate of CO_2 production[c]*
4.5 ± 0.3 (4.0–4.9)	0.13 ± 0.02 (0.10–0.18)	0.078 ± 0.01 (0.063–0.096)	16.5 ± 2.0 (14.1–19.5)	14.2 ± 1.4 (12.4–16.3)

[a] Estimated from computer analysis of the curve for $^{14}CO_2$ release. [b] This value is based on the computed initial rate of $^{14}CO_2$ release, corrected for refixation of $^{14}CO_2$ in mesophyll cells (see text). [c] Calculated on the basis that the rate of CO_2 production from C_4 acids equals the net photosynthesis rate plus the rate of CO_2 leakage from bundle-sheath cells (see Fig. 1).

Besides giving the best fit, first-order curve and the initial rate of $^{14}CO_2$ release from leaves, the computer-based treatment of the data also provides the total asymptotic amount of $^{14}CO_2$ that would be released from leaves and the rate constant for the process generating the $^{14}CO_2$. For these experiments with maize the averages for these parameters were 0.13 ± 0.02 μmol CO_2 mg^{-1} Chl and 0.078 ± 0.01 s^{-1}, respectively (Table I). The average photosynthesis rate for the seven, separate experiments, measured from the rate of $^{14}CO_2$ fixation during the pulse period, was 4.5 ± 0.3 μmol min^{-1} mg^{-1} Chl. Possible relationships between these parameters and leak rates are discussed below.

Verification by an Alternative Procedure

The procedure for measuring CO_2 leakage by comparing the computed initial rate of $^{14}CO_2$ release in the chase with the net photosynthesis rate was verified by the following alternative method. Leaves were subjected to a standard, pulse-chase treatment, but additional leaves were stopped at the end of the pulse period. With the analysis of the total radioactivity in C_4 acids (malate plus aspartate) and the proportion in the C-4 carboxyl of these C_4 acids, the total ^{14}C lost from the C-4 carboxyl during the chase period could be determined. This provides a measure of the total $^{14}CO_2$ produced in bundle-sheath cells, the majority of which would appear in 3-phosphoglycerate, hexose phosphates, and the end products Suc and starch. This value was then compared with the total $^{14}CO_2$ released from bundle-sheath cells that was computed from the curve for $^{14}CO_2$ release from leaves.

The results of this experiment are presented in Table II. The leak rate, expressed as a percentage of the rate of CO_2 generation in bundle-sheath cells, was 16.4% using our simple standard procedure, based on the measurement of the initial velocity of $^{14}CO_2$ release. The analysis of the ^{14}C in C_4 acids gave a value of 7.95 × 10^6 cpm mg^{-1} Chl for total ^{14}C released from the C-4 carboxyl of C_4 acids in the chase period, whereas the total $^{14}CO_2$ leaked, corrected for $^{14}CO_2$ lost during transfer to the chase chamber or refixed in mesophyll cells, was 1.42 × 10^6 cpm mg^{-1} Chl. This gave a leak rate, expressed as a percentage of the measured $^{14}CO_2$ produced from C_4 acids, of 17.9%, which is close to the value of 16.4% obtained by our standard procedure. The latter procedure was adopted for routine studies, since it avoided the complex and time-consuming analysis of the radioactivity in the C-4 carboxyl of C_4 acids.

Dark Controls and Photorespiratory CO_2

As shown previously (Hatch and Slack, 1966), the transfer of ^{14}C from the C-4 carboxyl of C_4 acids essentially ceases when sugarcane leaves are subject to a $^{12}CO_2$ chase in the dark. When we exposed maize leaves to a $^{14}CO_2$ pulse in the light but then followed this with a chase treatment in the dark, the observed initial rate of $^{14}CO_2$ release was about 17% of that observed in the light. However, in the normal light treatment, about 85% of the $^{14}CO_2$ released from C_4 acids is refixed via Rubisco (average from the present study), whereas in the dark, there should be

Table II. *Comparison of methods for measuring CO_2 leakage from bundle-sheath cells in maize leaves*

The standard procedure based on measuring the initial velocity of $^{14}CO_2$ release (see text) is compared with an estimate of CO_2 leak based on the estimation of total $^{14}CO_2$ released in the chase and the loss of ^{14}C from the C-4 carboxyl of C_4 acids determined directly during the chase (see "Materials and Methods"). All analyses were conducted on the same set of two leaves.

CO_2 leak based on initial velocity of $^{14}CO_2$ released	
Net photosynthesis rate ($^{14}CO_2$ assimilated)	4.5 μmol min^{-1} mg^{-1} Chl
Initial rate of $^{14}CO_2$ release (computed from curve)	0.72 μmol min^{-1} mg^{-1} Chl
CO_2 leak (% of rate of CO_2 released)[a]	16.4%
CO_2 leak based on total $^{14}CO_2$ released	
$^{14}CO_2$ released in chase chamber (35.8 s)	0.91 × 10^6 cpm mg^{-1} Chl
Total $^{14}CO_2$ released (including $^{14}CO_2$ released during 3.3-s transfer period)	1.14 × 10^6 cpm mg^{-1} Chl
Total $^{14}CO_2$ leaked (corrected for CO_2 refixed in mesophyll)[b]	1.42 × 10^6 cpm mg^{-1} Chl
Total ^{14}C released as CO_2 from the C-4 carboxyl of C_4 acids[c]	7.95 × 10^6 cpm mg^{-1} Chl
Total $^{14}CO_2$ leaked as % of the $^{14}CO_2$ released from C_4 acids	17.9%

[a] Calculated in the usual way; see footnotes b and c in Table I. [b] Correction based on estimate of $^{14}CO_2$ that would be refixed via PEP carboxylase in mesophyll cells with 1000 μL L^{-1} external CO_2 and other standard conditions (see text and "Materials and Methods"). [c] ^{14}C released from the C-4 carboxyl of C_4 acids was determined from the analysis of the decline in ^{14}C in the C-4 of malate and aspartate during the total chase period of 39.1 s (see "Materials and Methods" for further details).

very little refixation of $^{14}CO_2$ released from C_4 acids (or any other source). Hence, a much greater proportion of the total $^{14}CO_2$ generated should be released.

To assess this potential refixation, we measured $^{14}CO_2$ fixation rates in leaves that were darkened after a period in the light. The initial rate during the first 10 s in the dark was about 16% of the rate observed in illuminated leaves, rapidly declining to about 4% after 45 s in the dark (close to the rate normally seen with darkened leaves). From these data it can be calculated that the rate of $^{14}CO_2$ production in leaves during the dark chase, following a light pulse in $^{14}CO_2$, would be at a maximum of about 3% of the rate of production in illuminated leaves.

The release of $^{14}CO_2$ in a dark chase following a $^{14}CO_2$ pulse in the dark was also measured. In this case the rate of $^{14}CO_2$ fixation was about 0.2 μmol min^{-1} mg^{-1} Chl, and the rate of $^{14}CO_2$ release in the chase, with 1000 μL L^{-1} $^{12}CO_2$, was about 4% of that observed in the standard light treatment. Essentially all of the fixed ^{14}C in the dark was located in the C-4 carboxyl of C_4 acids and the observed rate of release indicates a surprisingly high turnover of this C_4 acid pool labeled by dark fixation of $^{14}CO_2$, presumably via PEP carboxylase.

When leaves of the C_3 plant wheat were subjected to our standard pulse-chase treatment, a relatively rapid rate of $^{14}CO_2$ release from leaves was observed (Fig. 4). It seemed likely that this $^{14}CO_2$ was derived from photorespiration, and this was confirmed by showing that it was largely abolished by conducting the chase in 2% O_2 instead of normal air. By contrast, when maize leaves were subjected to a chase in 2% O_2, the kinetics of $^{14}CO_2$ released and the calculated leak rate were very similar to those observed in 21% O_2 (Fig. 4).

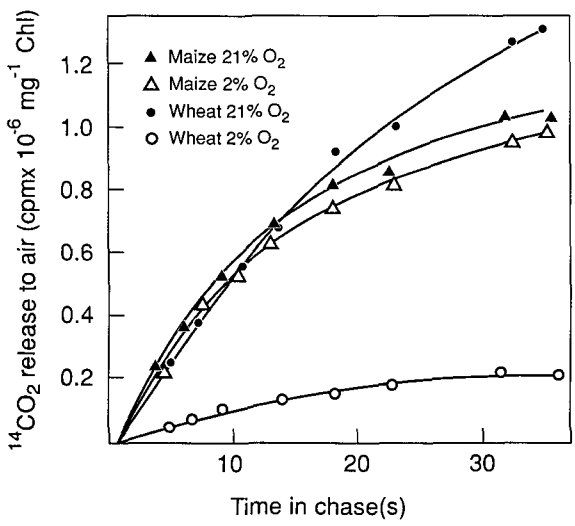

Figure 4. Kinetics of $^{14}CO_2$ released from maize (C_4) and wheat (C_3) leaves during a chase in $^{12}CO_2$ with either 21 or 2% O_2. Otherwise, the standard pulse-chase conditions applied (see text and "Materials and Methods"). The data are adjusted to allow for differences in the specific radioactivity of the $^{14}CO_2$ provided in the pulse stage (adjusted to a common specific radioactivity of 10.2×10^6 cpm/μmol $^{14}CO_2$).

Effect of Varying Growth Conditions and Leaf Age

The CO_2 leak rate of the third leaf of maize was measured in plants grown in a glasshouse under conditions varying from mid-summer (28/20°C, day/night) to mid-winter (25/15°C, day/night) when the total daily light dose would vary by more than a factor of 3. Under summer conditions, the third leaf was fully expanded in about 13 d after germination, whereas it took about 20 d under the winter conditions. However, the leak rate of the third leaf was not significantly different between these summer-grown (14.3 ± 1.4%) and winter-grown (13.5 ± 1.3%) plants.

The CO_2 leak rate of leaves 2, 3, and 4 of maize plants was determined when each leaf was just fully expanded (Table III). These analyses were conducted on the same day using plants germinated at different times, and very similar leak rates were observed. Similar leak rates were observed also for the third leaf of maize harvested either just prior to full expansion (10 d) or at full expansion (13 d), but the leak rate for an older third leaf (17 d from germination) was higher (Table III).

Survey of CO_2 Leak Rates in C_4 Species

The CO_2 leak rate was determined for several species of Gramineae representing the three different C_4 subgroups (Table IV). The values given are for the leak of $^{14}CO_2$ expressed as a percentage of the calculated rate of CO_2 production in bundle-sheath cells. The average CO_2 leak rate determined for the NADP-ME-type species was marginally higher than the rates for PEP carboxykinase-type and NAD-ME-type species, but the extremes of the range of average leak rates were only 8.3 to 14.2%.

For the NAD-ME-type dicotyledonous species *Atriplex spongiosa* and *Amaranthus edulis*, the CO_2 leak rate fell within the range observed for the grass species (Table V). However, for *Amaranthus* the curve for $^{14}CO_2$ release was generally biphasic, with points fitting a first-order curve up to about 20 s of the chase, followed by a second burst of $^{14}CO_2$ release. We have no explanation for this behavior, which was unique to the *Amaranthus* species. In contrast to *Amaranthus* and *Atriplex*, the leak of $^{14}CO_2$ observed for the two *Flaveria* species was exceptionally high. The average rates of 26 to 28% were about twice the values obtained for any other C_4 species, and the maximum individual rate recorded was 35%. C_4 *Flaveria* are NADP-ME-type species characterized by decarboxylating malate in bundle-sheath cells via a chloroplast-located NADP-specific malic enzyme. However, unlike the grass species belonging to this C_4 subgroup, the chloroplasts are located against the inner wall of bundle-sheath cells (centripetal) and the cell wall apparently lacks a suberin lamella (S. Craig, personal communication). By contrast, the NADP-ME-type grasses show an extreme centrifugal location of bundle-sheath chloroplasts and a suberized cell wall (Prendergast et al., 1987). When plant material becomes available, we will examine other dicotyledonous NADP-ME-type species to determine whether this high leak rate is a common feature of this particular group and the possible implications of this.

Table III. *Effect of varying leaf number or leaf maturity on the CO_2 leak rate of maize leaves*

Leaf No. and Plant Age (d)	Photosynthesis Rate [a]	CO_2 Leak Rate[b]
	μmol CO_2 min^{-1} mg^{-1} Chl	*% of rate of CO_2 production*
Experiment 1		
Leaf 2, 9	4.1	16.7
Leaf 3, 13	4.6	15.4
Leaf 4, 17	4.6	16.7
Experiment 2		
Leaf 3, 10	4.4	13.1
Leaf 3, 13	4.4	13.4
Leaf 3, 17	3.9	18.5

[a] From the rate of $^{14}CO_2$ fixation in the pulse. [b] Leak as a percentage of the rate of CO_2 released from C_4 acids in bundle-sheath cells (see Table I, footnote c).

For all of the C_4 species examined for a CO_2 leak rate, we also determined the net photosynthesis rate, the total pool of $^{14}CO_2$ released in the chase, and the rate constant for the $^{14}CO_2$ leakage process (see Table I for *Z. mays* data). We found no clear correlation between the CO_2 leak and either the leaf photosynthesis rate or the total amount of $^{14}CO_2$ released. An exception was *Flaveria* in which the very high leak rates were accompanied by high total amounts of $^{14}CO_2$ released, 2 to 3 times those observed for the other species (results not shown). Higher leak rates were generally associated with higher rate constants. Of course, the rate constant for $^{14}CO_2$ production would be a function of the size of the steady-state C_4 acid pool from which $^{14}CO_2$ is derived and the ongoing rate of flux through this C_4 acid pool (Fig. 1).

CONCLUDING REMARKS

As noted in the introduction, deduced values for CO_2 leakage from bundle-sheath cells of C_4 leaves, expressed as a percentage of CO_2 released in these cells by C_4 acid decarboxylation, have varied from 15 to 50% (Farquhar, 1983; Jenkins et al., 1989b; Henderson et al., 1992). During the present studies we developed a procedure that provides a direct measure of this CO_2 leak and requires only a minor correction to give the total CO_2 that leaks from bundle-sheath cells. For all C_4 species tested except *Flaveria*, leak rates were low and occurred within a quite narrow range of about 8 to 15% of the CO_2 released in bundle-sheath cells. These rates would be marginally higher (9–16%) if one assumes a gradient between intercellular air spaces and mesophyll cells of about 75 $\mu L\ L^{-1}$ (see above). However, for two species of *Flaveria* we observed leak rates of more than twice these values. The values recorded for most species were similar to the global value of about 14% that we deduced by modeling the inorganic carbon status of bundle-sheath cells (Jenkins et al., 1989b) but substantially less than those determined by a procedure based on measuring carbon-isotope discrimination (Farquhar, 1983; Henderson et al., 1992). Using measurements of short-term isotope discrimination, Henderson et al. (1992) calculated leak rates in the range from 21 to 30% for several C_4 grass species and some dicotyledonous plants. At this point an explanation for the discrepancy between these results is not apparent.

High CO_2 leak rates would substantially reduce the efficiency of C_4 photosynthesis (Farquhar, 1983; Furbank et al., 1990). For instance, a leak rate of 50% (equivalent to 100% overcycling of the C_4 acid cycle; Furbank et al., 1990) would increase the quantum requirement by about 4.0 mol quanta/mol CO_2 fixed. Taking a theoretical calculated quantum requirement of 14.2 mol quanta/mol CO_2 (assuming partial Q cycle operation during photosynthetic electron transport; Furbank et al., 1990), this CO_2 leak rate would increase the quantum requirement from a value equivalent to the lowest actually observed for C_4 species to one about equal to the average observed for C_3 species in normal air. With a leak equivalent to the highest recorded in our studies (35% for *Flaveria*), the increase would be 2.2 mol quanta/mol CO_2. However, the CO_2 leak rates we recorded for the remaining species would have only a minor effect on the quantum requirement and, hence, the efficiency of C_4 photosynthesis.

Table IV. *Survey of CO_2 leak rates for C_4 grass species from different C_4 subgroups*

Subgroup and Species	CO_2 Leak Rate[a]
	% of rate of CO_2 production
NADP-ME type	
Z. mays (7)	14.2±1.4
Sorghum bicolor	13.9,14.4
Echinochloa crus-galli	10.6,11.0
PEP carboxykinase type	
Urochloa panicoides	12.0,10.5
Panicum maximum (3)	10.4±2.3
Chloris gayana	12.1,7.8
NAD-ME type	
Panicum miliaceum (3)	8.3±1.1
Eleusine coracana	8.8,10.7
Panicum fluviicola	9.0

[a] Leak rate is corrected for $^{14}CO_2$ fixed via PEP carboxylase (see text) and expressed as a percentage of the rate of CO_2 production from C_4 acids (see Table I, footnote d). When one or two determinations were made, the individual results are given. Otherwise, results are given as the means ± SE, and the number of determinations is shown in parentheses.

Table V. *Survey of CO_2 leak rates for C_4 dicotyledonous species from different C_4 subgroups*

Subgroup, Species, and No. of Determinations	Average CO_2 Leak Rate[a]
	% of rate of CO_2 production
NADP-ME type	
Flaveria bidentis (4)	28.8 ± 4.6
F. trinervia (3)	26.0 ± 4.3
NAD-ME type	
Atriplex spongiosa (3)	12.3 ± 1.8
Amaranthus edulis (3)	9.1 ± 1.3[b]

[a] See footnote in Table IV for details. [b] Calculated from the first-order component of the curve obtained for $^{14}CO_2$ release during the first 20 s of the chase period (see text).

With regard to the high CO_2 leak rates observed for *Flaveria*, it may be significant that *Flaveria trinervia* leaves give a low quantum yield of only about 0.051 mol CO_2/mol quanta at 30°C (Monson et al., 1986). This contrasts with an average value of 0.065 mol CO_2/mol quanta for several NADP-ME-type grasses and 0.06 for dicotyledonous plants (*Euphorbia* species) belonging to this C_4 subgroup (Ehleringer and Pearcy, 1983).

ACKNOWLEDGMENTS

We wish to thank Drs. Graham Farquhar and Susanne von Caemmerer for their discussions and critical comments on this work.

Received September 26, 1994; accepted January 4, 1995.

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