Inorganic Carbon Diffusion between C₄ Mesophyll and Bundle Sheath Cells

Direct Bundle Sheath CO₂ Assimilation in Intact Leaves in the Presence of an Inhibitor of the C₄ Pathway

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ABSTRACT

Photosynthesis rates of detached Panicum miliaceum leaves were measured, by either CO₂ assimilation or oxygen evolution, over a wide range of CO₂ concentrations before and after supplying the phosphoenolpyruvate (PEP) carboxylase inhibitor, 3,3-dichloro-2-(dihydroxyphosphinoyl-methyl)-propenoate (DCDP). At a concentration of CO₂ near ambient, net photosynthesis was completely inhibited by DCDP, but could be largely restored by elevating the CO₂ concentration to about 0.8% (v/v) and above. Inhibition of isolated PEP carboxylase by DCDP was not competitive with respect to HCO₃⁻, indicating that the recovery was not due to reversal of enzyme inhibition. The kinetics of ¹³C-incorporation from ¹³CO₂ into early labeled products indicated that photosynthesis in DCDP-treated P. miliaceum leaves at 1% (v/v) CO₂ occurs predominantly by direct CO₂ fixation by ribulose 1,5-bisphosphate carboxylase. From the photosynthesis rates of DCDP-treated leaves at elevated CO₂ concentrations, permeability coefficients for CO₂ flux into bundle sheath cells were determined for a range of C₄ species. These values (6–21 micromoles per minute per milligram chlorophyll per micromolar, or 0.0016–0.0056 centimeter per second) were found to be about 100-fold lower than published values for mesophyll cells of C₃ plants. These results support the concept that a CO₂ permeability barrier exists to allow the development of high CO₂ concentrations in bundle sheath cells during C₄ photosynthesis.

It has been inferred that in C₄ species there must be a barrier to diffusion of CO₂ between bundle sheath and mesophyll cells (11, 12). This restriction to diffusion may be associated with the suberized lamellae (13), or related structures (25), seen in electron micrographs of bundle sheath cell walls of C₄ plants. Such a barrier would be necessary for the development of a relatively high CO₂ concentration in the bundle sheath cells during C₄ photosynthesis. The resulting suppression of oxygenase activity and associated reduction in photosynthesis account for many of the special physiological features of C₄ species (3, 11).

The resistance to diffusion of CO₂ from bundle sheath to mesophyll has not been directly measured, though considerations of quantum yields suggest that it must be large enough to prevent more than about 50% leakage of inorganic carbon and hence overcycling of the C₄ acid pathway relative to the rate of net assimilation (6). It is difficult to conceive an experimental method to examine the permeability properties of the bundle sheath cells in intact leaves during steady state C₄ photosynthesis. However, if the C₄ acid cycle could be rendered inoperative an approach seems feasible. This would involve blocking the C₄ acid cycle then elevating the CO₂ concentration in the mesophyll cells to such an extent that there would be sufficient direct flux of CO₂ into the bundle sheath cells to allow the direct assimilation of CO₂ by Rubisco.

A selective inhibitor of C₄ photosynthesis, DCDP, was recently described (15, 16). This compound, a PEP analog which inhibits PEP carboxylase, completely inhibits photosynthesis by C₄ leaves but has relatively little effect on C₃ leaves (15). We considered that this inhibitor may be suitable for studies on CO₂ diffusion, as outlined above. In the present study we show that higher than ambient CO₂ concentrations can largely restore photosynthesis in C₄ leaves when the C₄ pathway is inhibited. From these experiments it was possible to derive values for the permeability coefficient for CO₂ diffusion into bundle sheath cells.

MATERIALS AND METHODS

Chemicals

Biochemicals and reagent enzymes were obtained from Sigma Chemical Co. or Boehringer Mannheim, Australia. DCDP was synthesised at CSIRO and isolated as the monocyclohexylammonium salt (20). Solutions of the free acid of DCDP were obtained by passing solutions of the salt through small columns of cation exchange resin in the H⁺-form (Dowex-50), and then neutralising with dilute KOH.

Plant Material

Seedlings were grown in sterile soil, in a glasshouse with the temperature maintained between 20 and 30°C, under

1 Supported by a QEII Fellowship.

2 Abbreviations: Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; DCDP, 3,3-dichloro-2-(dihydroxyphosphinoylmethyl)-propenoate; PEP, phosphoenolpyruvate; PGA, 3-phosphoglycerate; PCR, photosynthetic carbon reduction.
natural illumination. Leaves of grass species were detached from 2 to 4 week-old plants, and then the basal part immediately recut under water. The top of the leaf was also usually cut off leaving a section about 13 cm long. For *Amaranthus edulis* the stem was cut diagonally under water several cm below the leaf to be used, and other leaves on that section of stem removed.

**Photosynthesis Measurements**

Photosynthesis rates were measured at 28 ± 2°C by gas-exchange using a clamp-on leaf chamber (PLC [B]; Analytical Development Co. Ltd). Usually the detached leaf, with the cut base in water, was clamped so that a 2.5 cm long portion (total area 2–6 cm²) was enclosed in the chamber. At CO₂ concentrations of 0.1% (v/v) and below, rates were measured in an open system as CO₂ uptake using an infrared gas analyser (LCA-2). For measurements at higher CO₂ concentrations, 0.5% (v/v) and above, the rate of photosynthetic oxygen evolution was measured using the same leaf chamber in a closed system. By connecting the inlet and outlet of the leaf chamber to an oxygen electrode chamber (Rank Bros., Cambridge, U.K.) via a system of 3-way taps it was possible to operate the system in either the open or closed mode and to switch easily between them without disturbing the leaf. The total volume of the closed system was 30.9 mL, determined from the decrease in oxygen concentration when 5 mL of air in the system was replaced by 5 mL of nitrogen. Despite the relatively large volume, the leaf chamber fan circulated the enclosed air through the leaf chamber and oxygen electrode chamber at a rate sufficient to prevent any significant lags in oxygen concentration measurements. Since O₂ evolution was measured at relatively high CO₂ concentrations a modified electrolyte at pH 9.0 was used similar to that used for leaf-disc electrodes (2) except that the final concentration of borate buffer was 0.5 M. With suitable amplification and offset, it was possible to measure easily increases in oxygen concentration of 0.04% (v/v) per min due to photosynthetic oxygen evolution by a 2.5 cm long leaf section in this system. Rates were usually recorded over a 5 to 10 min period during which oxygen concentration increased by less than 1% (v/v). Air was supplied to the system from cylinders containing either normal (0.035% v/v) or 0.1% (v/v) CO₂. For higher CO₂ concentrations, pure CO₂ was mixed with normal air, and humidified by passing over wet filter paper. Humid air was required for oxygen evolution measurements to reduce apparent changes in oxygen concentration when switching over from an open to a closed system.

Illumination (routinely 1600 μmol quanta m⁻² s⁻¹) was provided by an incandescent lamp using a glass dish of water between the chamber and the lamp as an additional heat filter. To vary the light intensity the distance of the lamp from the chamber was altered and shade-cloth filters were used. RH and air temperature were measured by the in-built chamber sensors and leaf temperature by a differential thermocouple system. The usual procedure for photosynthesis measurements was to clamp the leaf section in the chamber and continuously follow CO₂ assimilation while supplying (200–400 mL min⁻¹) normal air or air containing 0.1% (v/v) CO₂ as required. For measurements at higher CO₂ concentrations the leaf chamber was flushed (100 mL min⁻¹) with air containing the appropriate CO₂ concentration and after a period of equilibration of at least 15 min the system closed and the oxygen evolution rate measured. Rates were measured in this manner at least twice with the system flushed in between. For DCDP treatment, the inhibitor was added to the water supplied to the cut leaf base (4 mM final concentration). This was done under normal air conditions to allow continuous monitoring of inhibition of photosynthesis.

**Leaf ¹⁴CO₂ Labeling and Analysis of Labeled Metabolites**

Eight detached leaves were selected for uniformity and placed in a perspex leaf chamber (volume 2.32 L) equipped with mixing fans (7). The basal end of each leaf was immersed in an Eppendorf tube containing 1.5 mL of either water (controls) or 4 mM DCDP solution. The leaves in the chamber were illuminated with a 400 W Phillips HLGR lamp (about 800 μmol quanta m⁻² s⁻¹ at the leaf surface) and flushed with dry air at about 1.5 L min⁻¹. Air leaving the chamber contained 320 μL CO₂ L⁻¹. After 60 to 90 min, photosynthesis rates of individual leaves were checked by quickly removing them to the gas-exchange leaf chamber described above (under similar irradiance as the labeling chamber) and measuring CO₂ assimilation in normal air, then returning them to the labeling chamber. When it was established that net photosynthesis in the DCDP-treated leaves was completely inhibited, air containing 1% (v/v) CO₂ was supplied to the labeling chamber (1.0 L min⁻¹). After a further 30 min to allow the leaves to reach steady-state photosynthesis under these conditions, the labeling experiment was begun by sealing the chamber and injecting about 0.5 mCi of ¹⁴CO₂ gas (12 M). At timed intervals, individual DCDP-treated and control leaves were removed through a rubber gasket and immediately killed by plunging into 50 mL of boiling 80% (v/v) aqueous ethanol. Boiling was continued for several min then the leaf extracts allowed to cool. The specific radioactivity of the ¹⁴CO₂ was determined as described previously (7).

For extraction, leaf sections were removed from the original 80% (v/v) ethanol extract, ground in a mortar and pestle with 20 mL of 50% (v/v) aqueous ethanol, and the resulting mixture heated to 50°C for 10 min. After centrifugation (5000 g, 10 min) of this mixture the supernatant was pooled with the original 80% (v/v) ethanol extract. Residual solid material was then twice further extracted with 10 mL portions of water by the same method and liquid extracts pooled. Portions of the solid residues (containing insoluble "CO₂-labeled starch") were filtered onto glass fiber discs and the radioactivity determined by scintillation counting. The pooled ethanolic solutions were twice extracted with 20 mL chloroform to remove lipids and then reduced in volume on a rotary evaporator at 50°C under reduced pressure. The solutions were quantitatively transferred to Eppendorf tubes, made up to 1 mL, and aliquots removed to determine radioactivity, then dried under an air stream overnight. These dried residues were dissolved in small volumes of water (30–160 μL), centrifuged to remove any insoluble material, and stored frozen. Samples of these solutions were chromatographed on Whatman 3MM paper using 2-butanol:formic acid:water (6:1:2, v/v
v) (7) and the proportions of radioactivity in combined sugar-P, sucrose, PGA, aspartate, alanine, and malate determined using a radiochromatogram scanner. In this system the peak of radioactivity associated with PGA may also contain some triose-P. The proportion of sucrose was also determined as glucose and fructose following invertase treatment. Incorporation of $^{14}$C into metabolites was calculated on a leaf area basis and as a percentage of total incorporation.

**PEP Carboxylase Assays on Leaf Extracts**

Extracts from illuminated Panicum miliaceum leaves were prepared and PEP carboxylase assayed as described previously (15). For determining the effect of DCDP on activity at various HCO$_3^-$ concentrations the endogenous HCO$_3^-$ was decreased by flushing the assay mix with CO$_2$-free air. Remaining HCO$_3^-$ was removed from individual assays by allowing the PEP carboxylase reaction to run for 5 min before initiating the reaction with the appropriate HCO$_3^-$ concentration. The original NADH concentration was 0.3 mM. DCDP was added after measuring the control rate for 1 to 2 min in the absence of the inhibitor.

**Determination of Chl**

After gas-exchange measurements the exposed leaf section was cut from the leaf and homogenised in methanol. After centrifugation Chl was estimated spectrophotometrically according to the procedure of Mackinney (19). Chl in leaf extracts used for PEP carboxylase assays was determined in 90% acetone extracts according to Jeffrey and Humphrey (14).

**RESULTS AND DISCUSSION**

**Measurement of Photosynthesis Rates**

With available equipment it was not possible to measure photosynthesis rates by the same technique over the wide range of CO$_2$ concentrations required. Intact leaf photosynthesis at around ambient CO$_2$ levels is usually measured by CO$_2$ assimilation using open infrared gas analysis systems, whereas at very high CO$_2$ concentrations (1–5% [v/v]) CO$_2$ photosynthesis of leaf discs has been measured by oxygen evolution in closed, low-volume oxygen electrode chambers (2, 23). To measure photosynthesis of a single, detached leaf we devised a system which combines these techniques allowing rates to be determined at low CO$_2$ concentrations (0.1% [v/v] and below) by CO$_2$ assimilation and at high concentrations (0.5% [v/v] and above) by oxygen evolution (see “Materials and Methods”). To check whether these procedures gave comparable values, the photosynthesis rates of a single leaf were measured by either CO$_2$ assimilation or O$_2$ evolution with near-saturating CO$_2$ concentrations (0.07% [v/v] and 1% [v/v] CO$_2$, respectively) but low irradiances. Under these conditions we assume photosynthesis will be limited only by light so that rates of CO$_2$ assimilation (or oxygen evolution) should be similar regardless of the CO$_2$ concentration. The rates determined by the two procedures were generally in good agreement (Fig. 1). Over a range of limiting light intensities oxygen evolution rates parallel CO$_2$ assimilation rates but remain higher by about 2 μmol m$^{-2}$ s$^{-1}$. The higher oxygen evolution may be due to the fact that photosynthetic electron transport also provides ATP and reducing equivalents for other processes in addition to CO$_2$ fixation (e.g. nitrate reduction, sulphur assimilation).

**DCDP Inhibition of Photosynthesis and Recovery in High CO$_2$ Concentration**

Earlier studies showed that when the PEP carboxylase inhibitor, DCDP, was supplied to leaves at 1 mM via the transpiration stream, net photosynthesis of C$_4$ species was inhibited virtually completely after several hours. In contrast, photosynthesis in C$_3$ species was only partially inhibited (10–40%) by this compound (15). With C$_4$ leaves inhibited by DCDP, increasing the CO$_2$ concentration to 0.1% (v/v) resulted in only a very small increase in photosynthetic CO$_2$ assimilation (15). In the present work it was possible to test the effect of higher CO$_2$ concentrations by using oxygen evolution to measure photosynthesis.

In an experiment with a P. miliaceum leaf, control photosynthesis rates were measured at a range of CO$_2$ concentrations (Fig. 2) then DCDP supplied to the transpiration stream. To get rapid inhibition 4 mM DCDP was used in all the studies reported here. When net photosynthesis was completely inhibited by DCDP at atmospheric CO$_2$ concentration, the CO$_2$ concentration was elevated in gradual steps and
photosynthetic oxygen evolution rates were measured (Fig. 2). The results show that in the presence of DCDP photosynthesis was progressively recovered by increasing CO2 concentration. The photosynthesis rate at 5% (v/v) CO2 was about 60% of the control rate and this concentration was close to CO2 saturation for DCDP-treated tissue. Since there was a slow decline in control rates in the time period required for this experiment (several hours), the recovery of photosynthesis may have been greater than 60%. To check that the recovery of photosynthesis was not due to increasing inhibitor removal from the leaf tissue (even though inhibitor was supplied to the leaf throughout the experiment) the CO2 concentration in the air supply was finally decreased to 0.1% (v/v). A negative net photosynthesis rate was recorded (Fig. 2) indicating that DCDP continued to effectively inhibit C4 photosynthesis throughout the experiment. Rates of CO2 exchange at 0.1% (v/v) CO2 and below were similar to dark respiration rates, indicating that photosynthesis was virtually abolished by DCDP. One likely explanation for the high CO2-induced recovery of photosynthesis in DCDP treated tissue is that atmospheric CO2 diffuses directly into bundle sheath cells where it is fixed by Rubisco. Another possibility is dealt with in the following section.

**Effect of Bicarbonate on DCDP Inhibition of PEP Carboxylase**

To test if the recovery of photosynthesis in DCDP-inhibited leaves by high CO2 concentration could be due to a competitive effect of bicarbonate with DCDP, the effect of this inhibitor on PEP carboxylase activity in extracts of illuminated *P. miliaceum* leaves was examined. Bicarbonate was used at either a saturating concentration or at a limiting concentration (50 μM) close to the Km for this substrate (1). The latter concentration would be close to that prevailing in vivo during photosynthesis (assuming equilibrium with 4 μM CO2 at pH 7.4; see refs. 7 and 17). The extent of inhibition by DCDP was virtually the same at each HCO3− concentration (Fig. 3) indicating that recovery of photosynthetic activity in high CO2 concentration is unlikely to be due to reversal of PEP carboxylase inhibition by high HCO3− concentration.

**Labeling Kinetics for 14CO2 incorporation into DCDP-treated Leaves**

To determine whether the recovery of photosynthesis in DCDP-treated leaves at high CO2 concentration was due to direct fixation of atmospheric CO2 by Rubisco in bundle sheath cells, we examined the 14CO2 labeling pattern of leaves under these conditions. After treatment of *P. miliaceum* leaves with DCDP in normal air they were allowed to recover photosynthesis in air containing 1% [v/v] CO2. Then the leaves were exposed to 14CO2, rapidly killed after intervals, and the 14C-labeled metabolites analyzed. The kinetics of 14C-incorporation into metabolites for control leaves at 1% (v/v) CO2 (Fig. 4) is generally similar to that previously observed for leaves of NAD-ME-type C4 species in normal air (7). Combined C4 acids (malate plus aspartate) are rapidly labeled followed later by rapid labeling of PGA and then phosphor-ylated sugars of the PCR cycle. In contrast, the pattern of
labeling after DCDP treatment indicates that PGA is labeled first, with $^{14}$C appearing more slowly in C₄ acids, at about the time that sugar-P compounds are being labeled. This different pattern of labeling is more clearly seen when $^{14}$C-incorporations into labeled metabolites are plotted as a percentage of total incorporation (lower panels, Fig. 4). Extrapolation of the curves to zero time suggests that CO₂ is initially incorporated predominantly into C₄ acids in the control leaves whereas, after DCDP treatment, PGA is clearly the predominant (greater than 80%) initially labeled metabolite.

The results in Figure 4 demonstrate convincingly that photosynthesis in DCDP-treated leaves at high CO₂ concentration occurs mainly via direct fixation of atmospheric CO₂ by Rubisco in bundle sheath cells, with the C₄ acid cycle playing only a minor role. Interestingly, even in the control leaves at 1% (v/v) CO₂ the results suggest that there may be a minor proportion (up to 20%) of the assimilated CO₂ fixed directly via the PCR cycle, based on where the extrapolated curves for PGA plus products and C₄ acids cut the axis. By contrast, the labeling for C₃ leaves (including P. miliaceum) in normal air showed that the percentage incorporation into PGA plus products extrapolates to zero at zero time (10).

### Determination of Permeability Coefficients for CO₂ Flux into Bundle Sheath Cells

The permeability coefficient, $P_a$, is a constant which relates the rate of flux of a compound, $x$, across a membrane or other permeability barrier to the concentration gradient across that barrier (22). In recent work from this laboratory this constant has been defined for isolated bundle sheath cells as the 'Diffusion constant,' $K_D$ (11, 24). The studies described above show that, in the absence of a functional C₄ acid cycle, it is possible to generate a sufficient diffusive flux of CO₂ across the barrier between mesophyll cells and bundle sheath cells to support high rates of photosynthesis by elevating the CO₂ concentration. Thus, it is possible to derive a permeability coefficient for CO₂ diffusion into bundle sheath cells, $P_{CO₂}$, from the equation:

$$ \text{Rate of CO}_2 \text{ flux} = P_{CO₂} \left( [\text{CO}_2]^{\text{meso}} - [\text{CO}_2]^{\text{BS}} \right) $$

where the superscripts denote the CO₂ concentrations in the mesophyll and bundle sheath cells.

To determine $P_{CO₂}$, the rate of CO₂ flux is assumed to be equal to the steady-state photosynthesis rate at elevated CO₂ concentration in the presence of DCDP. Since it was not possible to determine intercellular CO₂ concentration, $[\text{CO}_2]^{\text{meso}}$ is calculated from its solubility assuming the CO₂ concentration in the intercellular spaces is the same as in the supplied air. Although this assumption introduces some error (since there must be a CO₂ gradient from the atmosphere to the intercellular spaces during photosynthesis), at the high CO₂ concentrations used to recover photosynthesis in DCDP-treated leaves this would not have a substantial effect. For example, calculations showed that for P. miliaceum the $P_{CO₂}$ value was only increased by about 30%, even assuming that the high CO₂ concentration caused stomatal conductance to be decreased by 90%. Experiments with a range of species have indicated that stomatal conductance is decreased by about this extent due to CO₂ concentrations of approximately 0.1% (v/v) (21, 23) but may actually increase again at higher CO₂ concentrations (23).

To obtain $[\text{CO}_2]^{\text{BS}}$ it was assumed that, at high irradiances, Rubisco activity and hence photosynthesis rate are limited only by the CO₂ concentration in bundle sheath cells. Providing that the $V_{\text{max}}$ for Rubisco is known, it is possible to calculate $[\text{CO}_2]^{\text{BS}}$ from the enzyme kinetic equation:

$$ v = \frac{V_{\text{max}} [\text{CO}_2]^{\text{BS}}}{K_C (1 + [\text{O}_2]^{\text{BS}} / K_O)} $$

where $K_C$ is the $K_M$ (CO₂), $K_O$ is the inhibition constant for oxygen (0.8 mM; ref. 18) and $[\text{O}_2]^{\text{BS}}$ is the oxygen concentration in bundle sheath cells. Values of $K_C$ for Rubisco from individual C₄ species or C₄ subgroups were taken from published determinations (18, 26), corrected to account for the
effect of ionic strength on the pK of H₂CO₃ (see ref. 8). In the present work it was assumed that Vₘₐₓ is equal to the control photosynthesis rate at 5% CO₂. v is equal to the photosynthetic rate at elevated CO₂ concentration in the presence of DCDP, and that the [O₂] is at a concentration equivalent to its solubility in equilibrium with air. Although higher values of [O₂] may develop during photosynthesis (8, 17), assuming such higher values did not markedly affect the calculated Pₐ values.

The results of experiments to determine Pₐ with a range of C₄ species are presented in Table I. Experiments were conducted by a procedure similar to that described above for Figure 2, except photosynthesis rates were measured at fewer CO₂ concentrations. For each species net photosynthesis was completely inhibited after DCDP treatment; rates of net CO₂ evolution approached dark respiration rates. Subsequent elevation of the CO₂ concentration caused a recovery of photosynthesis in all species, but to variable extents. For most of the NAD-ME and PCK types the rate after DCDP treatment at about 5% (v/v) CO₂ approached the control rates. This indicates that DCDP has minimal effects other than inhibiting PEP carboxylase. For species of the NADP-ME subgroup the poorer recoveries of photosynthesis may partly reflect the disrupted C₄ acid cycle metabolism; in this subgroup the decarboxylation of malate in bundle sheath cells provides not only CO₂ but also at least half of the NADPH required for PGA reduction. Alternatively, the resistance to CO₂ diffusion into the bundle sheath cell in this subgroup may be greater than in the other C₄ subgroups as suggested elsewhere (see below).

Permeability coefficients were calculated on the assumption that the C₄ acid cycle is inhibited completely by DCDP. Since any residual C₄ acid cycle activity would diminish the assumed direct CO₂ flux from the mesophyll to the bundle sheath cells, these Pₐ values are maximal estimates. Only the values calculated at a CO₂ concentration of 1.6% (v/v), which is clearly a limiting concentration, are presented; permeability coefficients calculated from the photosynthesis rates at 5% (v/v) CO₂ were similar but lower by 20 to 50%. For convenience, the values are presented in the same rate units as used for photosynthesis (per mm concentration gradient) as well as in the more conventional cm s⁻¹ units (22). The former units are more appropriate for our purposes (e.g. modeling the inorganic carbon pool in C₄ leaves; see ref. 17). To allow easy conversion of rates and Pₐ values to a leaf area basis, as routinely used for gas-exchange data, Chl contents of the leaves are also presented. Permeability coefficients determined using intact leaves in

### Table I. Net Photosynthesis Rates at Several CO₂ Concentrations for Leaf Sections from a Range of C₄ Species Before and After DCDP Treatment, and Derived Bundle Sheath CO₂ Permeability Coefficients

<table>
<thead>
<tr>
<th>Species and C₄ Subgroup</th>
<th>Chl Content (mg m⁻²)</th>
<th>CO₂ Concentration (v/v)</th>
<th>Net Photosynthesis Rate Control (μmol min⁻¹ (mg Chl)⁻¹)</th>
<th>Net Photosynthesis Rate 4 mM DCDP (μmol min⁻¹ (mg Chl)⁻¹)</th>
<th>Permeability Coefficient, Pₐ (cm s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panicum miliaceum (NAD-ME)</td>
<td>280</td>
<td>0.026c</td>
<td>1.62</td>
<td>13.7</td>
<td>5.6 x 10⁻³</td>
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<tr>
<td></td>
<td></td>
<td>4.90</td>
<td>6.4</td>
<td>8.1</td>
<td></td>
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<tr>
<td>Amaranthus edulis (NAD-ME)</td>
<td>269</td>
<td>0.019c</td>
<td>1.62</td>
<td>6.4</td>
<td>1.8 x 10⁻³</td>
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<tr>
<td></td>
<td></td>
<td>5.05</td>
<td>12.0</td>
<td>5.1</td>
<td></td>
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<tr>
<td>Chloris gayana (PCK)</td>
<td>383</td>
<td>0.029c</td>
<td>1.61</td>
<td>4.6</td>
<td>2.7 x 10⁻³</td>
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<tr>
<td></td>
<td></td>
<td>5.00</td>
<td>11.0</td>
<td>4.2</td>
<td></td>
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<tr>
<td>Urochloa panicoides (PCK)</td>
<td>243</td>
<td>0.030c</td>
<td>1.71</td>
<td>15.9</td>
<td>2.4 x 10⁻³</td>
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<tr>
<td></td>
<td></td>
<td>4.90</td>
<td>6.2</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>Panicum maximum (PCK)</td>
<td>373</td>
<td>0.027c</td>
<td>1.63</td>
<td>5.9</td>
<td>3.8 x 10⁻³</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.20</td>
<td>12.7</td>
<td>6.1</td>
<td></td>
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<tr>
<td>Echinochloa crusgalli (NADP-ME)</td>
<td>247</td>
<td>0.030c</td>
<td>1.59</td>
<td>5.0</td>
<td>2.4 x 10⁻³</td>
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<tr>
<td></td>
<td></td>
<td>5.10</td>
<td>12.8</td>
<td>4.0</td>
<td></td>
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<tr>
<td>Digitaria sanguinalis (NADP-ME)</td>
<td>372</td>
<td>0.024c</td>
<td>1.71</td>
<td>6.3</td>
<td>1.6 x 10⁻³</td>
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<td></td>
<td></td>
<td>5.21</td>
<td>11.5</td>
<td>2.7</td>
<td></td>
</tr>
</tbody>
</table>

* Chl content is given to allow conversion of photosynthesis rates and Pₐ values to a leaf area basis.  
* Calculated using values of bundle sheath cell surface area per mg leaf Chl determined in Ref. 12.  
* CO₂ concentration in air leaving the leaf chamber for control rates. For DCDP-inhibited leaf sections the leaving [CO₂] was similar to the [CO₂] in the supplied air (0.035% v/v).  
* Measured on another equivalent leaf section.
this study (6–21 μmol min⁻¹ [mg Chl]⁻¹ mm⁻¹ for a range of C₄ species) are in very good agreement with those determined by the different procedure using isolated bundle sheath cells in the accompanying paper (6–30 μmol min⁻¹ [mg Chl]⁻¹ mm⁻¹; ref. 8). In each case the lowest values were obtained with NADP-ME-type C₄ species and the highest for NAD-ME-types. From the combined results, average values (μmol min⁻¹ [mg Chl]⁻¹ mm⁻¹) for each C₄ subgroup are: NADP-ME, 7; PCK, 15, and NAD-ME, 22. These P₄₀ values are consistent with suggestions based on quantum yields and carbon isotope fractionation studies that NAD-ME-type bundle sheath cells may be the most leaky toward CO₂ and NADP-ME-types the least leaky, of the C₄ subgroups (6).

The values obtained for the CO₂ permeability coefficients for bundle sheath cells may also be compared to values obtained for C₃ mesophyll cells. Using various procedures the transfer resistances (essentially the inverse of the permeability coefficient) for C₃ mesophyll cells have been determined to be in the range 1.2 to 2.4 bar m² s⁻¹ mm⁻¹ (or about 6 cm⁻¹; 4, 5). Assuming 400 mg Chl m⁻² and 20°C, these values translate to a range of permeability coefficients from 3190 to 1595 μmol min⁻¹ [mg Chl]⁻¹ mm⁻¹ (or 0.14–0.071 cm s⁻¹). A rather higher permeability coefficient (0.35 cm s⁻¹) was obtained for the diffusion of CO₂ through lipid bilayer membranes (9). Therefore, the values we obtain for bundle sheath cells (6–21 μmol min⁻¹ [mg Chl]⁻¹ mm⁻¹, or 0.0016 to 0.0056 cm s⁻¹) are from 75 to 500 times lower than for C₃ mesophyll cells or up to 200 times lower than for lipid bilayers. This reflects the extent of the barrier to diffusion of CO₂ that C₄ bundle sheath cells have evolved to enable them to perform their specialised function in C₄ photosynthesis.

CONCLUDING COMMENTS

Data is presented which shows that, with the C₄ acid cycle specifically inhibited, it is still possible for C₄ leaves to carry out high rates of photosynthesis when provided with a sufficiently high CO₂ concentration. The relatively high concentrations of CO₂ required provide evidence that CO₂ diffusion between mesophyll and bundle sheath cells is highly restricted, as inferred from earlier studies (11). We determined permeability coefficients for CO₂ diffusion into bundle sheath cells which show these cells to be at least 100-fold less permeable to CO₂ than C₃ mesophyll cells. These permeability coefficient values are a critical component of quantitative models developed to describe the steady-state inorganic carbon status of C₄ species (17) and C₃ photosynthesis (e.g. ref. 6). The lipid-polymer, suberin, appears the most likely structural component responsible for the CO₂ diffusion barrier (see introduction) though this has yet to be unambiguously proven. It is also unknown whether the diffusion of other gases is restricted to the same extent. Especially significant in this regard is oxygen, photosynthetically produced in bundle sheath cells of the majority of C₄ species.

The experiments reported here also show that by elevating the CO₂ concentration it may be feasible to rescue C₄ plants which have an ineffective C₄ acid cycle. Hence, a search for mutants with lesions in the C₄ acid cycle could be undertaken. Whether photosynthesis can be maintained in the long-term under these conditions, however, needs to be established.

LITERATURE CITED