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Inorganic Carbon Diffusion between C_4 Mesophyll and Bundle Sheath Cells

Direct Bundle Sheath CO_2 Assimilation in Intact Leaves in the Presence of an Inhibitor of the C_4 Pathway

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ABSTRACT

Photosynthesis rates of detached *Panicum miliaceum* leaves were measured, by either CO_2 assimilation or oxygen evolution, over a wide range of CO_2 concentrations before and after supplying the phosphoenolpyruvate (PEP) carboxylase inhibitor, 3,3-dichloro-2-(dihydroxyphosphinoyl-methyl)-propenoate (DCDP). At a concentration of CO_2 near ambient, net photosynthesis was completely inhibited by DCDP, but could be largely restored by elevating the CO_2 concentration to about 0.8% (v/v) and above. Inhibition of isolated PEP carboxylase by DCDP was not competitive with respect to HCO_3^-, indicating that the recovery was not due to reversal of enzyme inhibition. The kinetics of ^{14}C-incorporation from $^{14}CO_2$ into early labeled products indicated that photosynthesis in DCDP-treated *P. miliaceum* leaves at 1% (v/v) CO_2 occurs predominantly by direct CO_2 fixation by ribulose 1,5-bisphosphate carboxylase. From the photosynthesis rates of DCDP-treated leaves at elevated CO_2 concentrations, permeability coefficients for CO_2 flux into bundle sheath cells were determined for a range of C_4 species. These values (6–21 micromoles per minute per milligram chlorophyll per millimolar, or 0.0016–0.0056 centimeter per second) were found to be about 100-fold lower than published values for mesophyll cells of C_3 plants. These results support the concept that a CO_2 permeability barrier exists to allow the development of high CO_2 concentrations in bundle sheath cells during C_4 photosynthesis.

It has been inferred that in C_4 species there must be a barrier to diffusion of CO_2 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 C_4 plants. Such a barrier would be necessary for the development of a relatively high CO_2 concentration in the bundle sheath cells during C_4 photosynthesis. The resulting suppression of oxygenase activity and associated reduction in photorespiration account for many of the special physiological features of C_4 species (3, 11).

The resistance to diffusion of CO_2 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 C_4 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 C_4 photosynthesis. However, if the C_4 acid cycle could be rendered inoperative an approach seems feasible. This would involve blocking the C_4 acid cycle then elevating the CO_2 concentration in the mesophyll cells to such an extent that there would be sufficient direct flux of CO_2 into the bundle sheath cells to allow the direct assimilation of CO_2 by Rubisco.[2]

A selective inhibitor of C_4 photosynthesis, DCDP, was recently described (15, 16). This compound, a PEP analog which inhibits PEP carboxylase, completely inhibits photosynthesis by C_4 leaves but has relatively little effect on C_3 leaves (15). We considered that this inhibitor may be suitable for studies on CO_2 diffusion, as outlined above. In the present study we show that higher than ambient CO_2 concentrations can largely restore photosynthesis in C_4 leaves when the C_4 pathway is inhibited. From these experiments it was possible to derive values for the permeability coefficient for CO_2 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

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[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 cm^2) was enclosed in the chamber. At CO_2 concentrations of 0.1% (v/v) and below, rates were measured in an open system as CO_2 uptake using an infrared gas analyser (LCA-2). For measurements at higher CO_2 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 O_2 evolution was measured at relatively high CO_2 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) CO_2. For higher CO_2 concentrations, pure CO_2 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 CO_2 assimilation while supplying (200–400 mL min^{-1}) normal air or air containing 0.1% (v/v) CO_2 as required. For measurements at higher CO_2 concentrations the leaf chamber was flushed (100 mL min^{-1}) with air containing the appropriate CO_2 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 $^{14}CO_2$ 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 CO_2 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 CO_2 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) CO_2 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 $^{14}CO_2$ gas (12 mL). 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 $^{14}CO_2$ 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 $^{14}CO_2$-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) (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 that 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

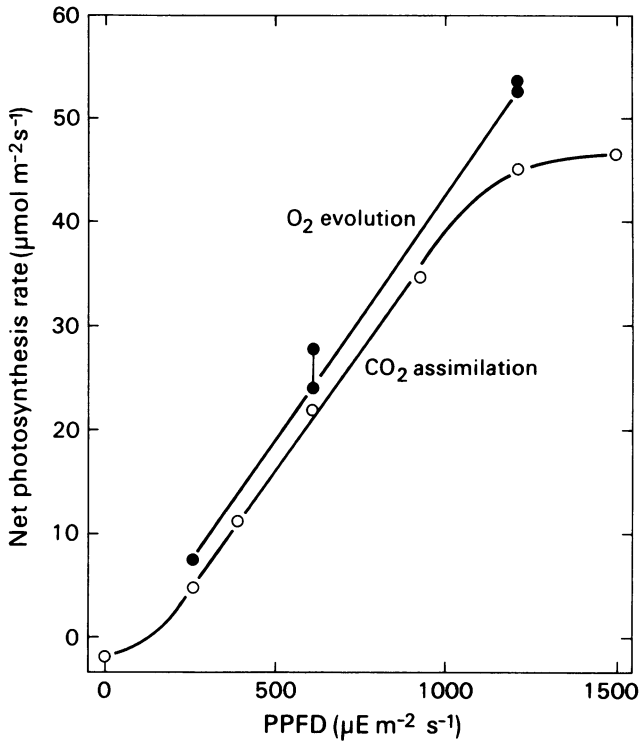

Figure 1. Responses of photosynthetic oxygen evolution and CO_2 assimilation of a *P. miliaceum* leaf to varying irradiance at near-saturating CO_2 concentration. Photosynthetic oxygen evolution rates, then CO_2 assimilation rates were measured at the irradiances shown (highest first). The CO_2 concentrations were 1% (v/v) for measuring O_2 exchange, and in the range 0.068 to 0.084% (v/v) for measuring CO_2 assimilation.

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

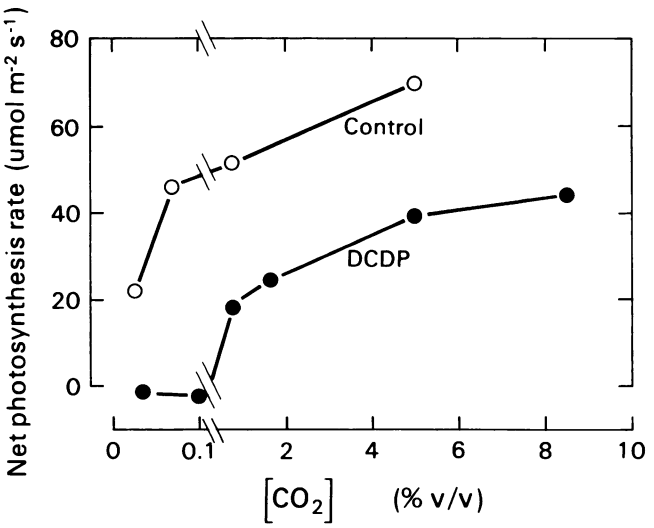

Figure 2. Effect of varying CO_2 concentration on photosynthesis rates of a *P. miliaceum* leaf inhibited by DCDP. Photosynthetic oxygen evolution (rates above 0.1% [v/v] CO_2) and CO_2 assimilation (rates at 0.1% [v/v] CO_2 and below) rates were measured on a leaf as described in "Materials and Methods." After measuring control rates at the CO_2 concentrations shown (highest first) the CO_2 concentration in the supplied air was returned to near-ambient (0.035% [v/v]) and, after a period of equilibration, the water supplied to the cut leaf base was replaced by 4 mM DCDP. When net photosynthesis was inhibited the CO_2 concentration was increased over the range 0.8 to 8.6% (v/v) in steps and rates measured again. The rate at 0.1% (v/v) CO_2 was measured last. The Chl content of the leaf section was 286 mg m^{-2}.

photosynthetic oxygen evolution rates were measured (Fig. 2). The results show that in the presence of DCDP photosynthesis was progressively recovered by increasing CO_2 concentration. The photosynthesis rate at 5% (v/v) CO_2 was about 60% of the control rate and this concentration was close to CO_2 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 CO_2 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 C_4 photosynthesis throughout the experiment. Rates of CO_2 exchange at 0.1% (v/v) CO_2 and below were similar to dark respiration rates, indicating that photosynthesis was virtually abolished by DCDP. One likely explanation for the high CO_2-induced recovery of photosynthesis in DCDP treated tissue is that atmospheric CO_2 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 CO_2 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 K_m for this substrate (1). The latter concentration would be close to that prevailing *in vivo* during photosynthesis (assuming equilibrium with 4 μM CO_2 at pH 7.4; see refs. 7 and 17). The extent of inhibition by DCDP was virtually the same at each HCO_3^- concentration (Fig. 3) indicating that recovery of photosynthetic activity in high CO_2 concentration is unlikely to be due to reversal of PEP carboxylase inhibition by high HCO_3^- concentration.

Labeling Kinetics for $^{14}CO_2$ Incorporation into DCDP-treated Leaves

To determine whether the recovery of photosynthesis in DCDP-treated leaves at high CO_2 concentration was due to direct fixation of atmospheric CO_2 by Rubisco in bundle sheath cells, we examined the $^{14}CO_2$ 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] CO_2. Then the leaves were exposed to $^{14}CO_2$, rapidly killed after intervals, and the ^{14}C-labeled metabolites analyzed. The kinetics of ^{14}C-incorporation into metabolites for control leaves at 1% (v/v) CO_2 (Fig. 4) is generally similar to that previously observed for leaves of NAD-ME-type C_4 species in normal air (7). Combined C_4 acids (malate plus aspartate) are rapidly labeled followed later by rapid labeling of PGA and then phosphorylated sugars of the PCR cycle. In contrast, the pattern of

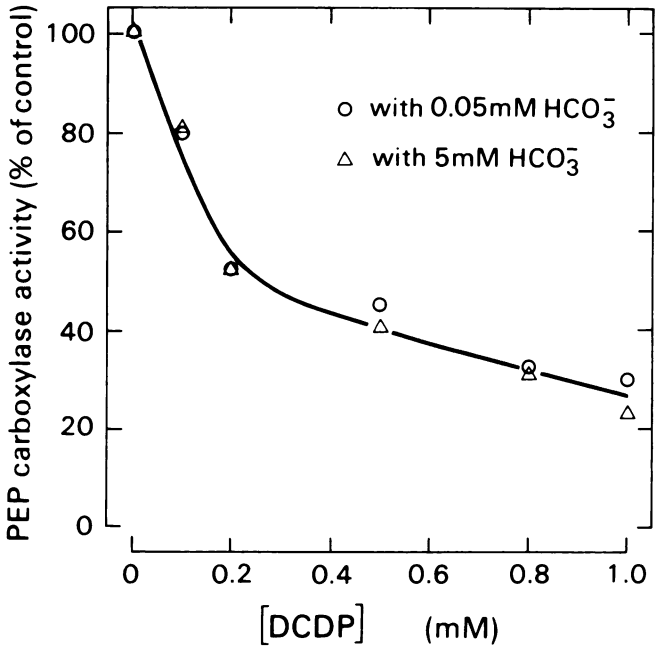

Figure 3. Effect of DCDP on PEP carboxylase activity in a *P. miliaceum* leaf extract at saturating and subsaturating concentrations of HCO_3^-. PEP carboxylase activities were measured in assays containing 5 mM PEP and the $KHCO_3$ and DCDP concentrations shown. Average control rates were 8.2 and 13.1 μmol min^{-1} (mg Chl)$^{-1}$, respectively.

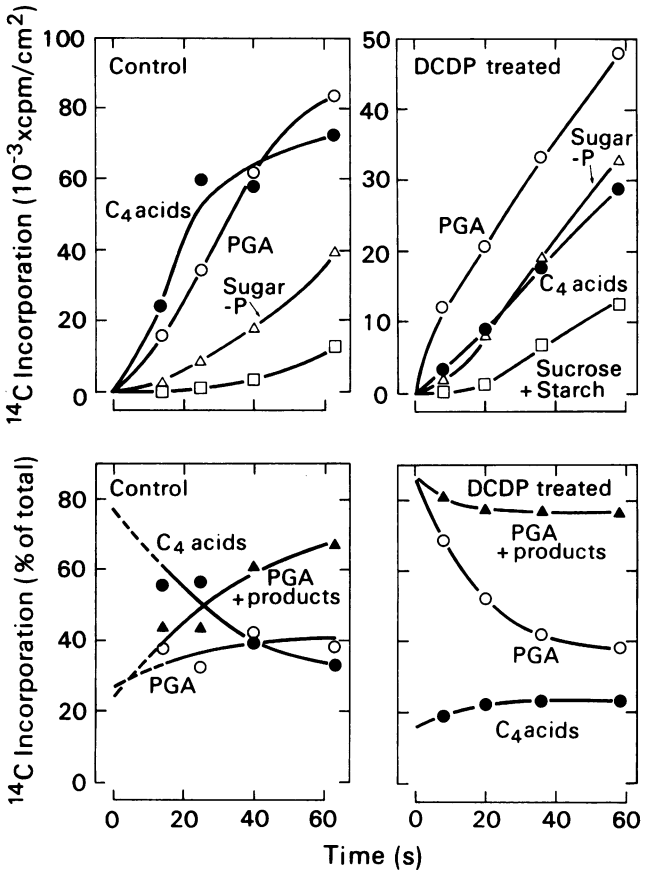

Figure 4. Kinetics of ^{14}C-labeling of photosynthetic metabolites during short-term exposure of control and DCDP-treated leaf sections of *P. miliaceum* to air containing 1% (v/v) $^{14}CO_2$. Detached, illuminated leaves were supplied with water or 4 mM DCDP to the cut base and flushed with normal air. When net photosynthesis was inhibited in the treated leaves (checked by measuring CO_2 assimilation of individual leaf sections), the air stream was supplemented with 1% (v/v) CO_2. After a further 30 min $^{14}CO_2$ was supplied and, after the times indicated, leaf sections were rapidly killed for analysis. For details see "Materials and Methods." Based on ^{14}C-incorporation, average photosynthesis rates were 29.0 and 17.1 μmol m^{-2} s^{-1} for control and DCDP-treated leaf sections, respectively.

labeling after DCDP treatment indicates that PGA is labeled first, with ^{14}C appearing more slowly in C_4 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 CO_2 is initially incorporated predominantly into C_4 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 CO_2 concentration occurs mainly via direct fixation of atmospheric CO_2 by Rubisco in bundle sheath cells, with the C_4 acid cycle playing only a minor role. Interestingly, even in the control leaves at 1% (v/v) CO_2 the results suggest that there may be a minor proportion (up to 20%) of the assimilated CO_2 fixed directly via the PCR cycle, based on where the extrapolated curves for PGA plus products and C_4 acids cut the axis. By contrast, the labeling for C_4 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 CO_2 Flux into Bundle Sheath Cells

The permeability coefficient, P_x, 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 C_4 acid cycle, it is possible to generate a sufficient diffusive flux of CO_2 across the barrier between mesophyll cells and bundle sheath cells to support high rates of photosynthesis by elevating the CO_2 concentration. Thus, it is possible to derive a permeability coefficient for CO_2 diffusion into bundle sheath cells, P_{CO_2}, from the equation:

$$\text{Rate of } CO_2 \text{ flux} = P_{CO_2}([CO_2]^{meso}-[CO_2]^{BS})$$

where the superscripts denote the CO_2 concentrations in the mesophyll and bundle sheath cells.

To determine P_{CO_2} the rate of CO_2 flux is assumed to be equal to the steady-state photosynthesis rate at elevated CO_2 concentration in the presence of DCDP. Since it was not possible to determine intercellular CO_2 concentration, $[CO_2]^{meso}$ is calculated from its solubility assuming the CO_2 concentration in the intercellular spaces is the same as in the supplied air. Although this assumption introduces some error (since there must be a CO_2 gradient from the atmosphere to the intercellular spaces during photosynthesis), at the high CO_2 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_{CO_2} value was only increased by about 30%, even assuming that the high CO_2 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 CO_2 concentrations of approximately 0.1% (v/v) (21, 23) but may actually increase again at higher CO_2 concentrations (23).

To obtain $[CO_2]^{BS}$ it was assumed that, at high irradiances, Rubisco activity and hence photosynthesis rate are limited only by the CO_2 concentration in bundle sheath cells. Providing that the V_{max} for Rubisco is known, it is possible to calculate $[CO_2]^{BS}$ from the enzyme kinetic equation:

$$v = \frac{V_{max}[CO_2]^{BS}}{[CO_2]^{BS} + K_C(1 + [O_2]^{BS}/K_O)}$$

where K_C is the K_M (CO_2), K_O is the inhibition constant for oxygen (0.8 mM; ref. 18) and $[O_2]^{BS}$ is the oxygen concentration in bundle sheath cells. Values of K_C for Rubisco from individual C_4 species or C_4 subgroups were taken from published determinations (18, 26), corrected to account for the

effect of ionic strength on the pK of H_2CO_3 (see ref. 8). In the present work it was assumed that V_{max} is equal to the control photosynthesis rate at 5% CO_2, v is equal to the photosynthetic rate at elevated CO_2 concentration in the presence of DCDP, and that the $[O_2]^{BS}$ is at a concentration equivalent to its solubility in equilibrium with air. Although higher values of $[O_2]^{BS}$ may develop during photosynthesis (8, 17), assuming such higher values did not markedly affect the calculated P_{CO_2} values.

The results of experiments to determine P_{CO_2} with a range of C_4 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 CO_2 concentrations. For each species net photosynthesis was completely inhibited after DCDP treatment; rates of net CO_2 evolution approached dark respiration rates. Subsequent elevation of the CO_2 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) CO_2 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 C_4 acid cycle metabolism; in this subgroup the decarboxylation of malate in bundle sheath cells provides not only CO_2 but also at least half of the NADPH required for PGA reduction. Alternatively, the resistance to CO_2 diffusion into the bundle sheath cell in this subgroup may be greater than in the other C_4 subgroups as suggested elsewhere (see below).

Permeability coefficients were calculated on the assumption that the C_4 acid cycle is inhibited completely by DCDP. Since any residual C_4 acid cycle activity would diminish the assumed direct CO_2 flux from the mesophyll to the bundle sheath cells, these P_{CO_2} values are maximal estimates. Only the values calculated at a CO_2 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) CO_2 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 C_4 leaves; see ref. 17). To allow easy conversion of rates and P_{CO_2} 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 CO_2 Concentrations for Leaf Sections from a Range of C_4 Species Before and After DCDP Treatment, and Derived Bundle Sheath Cell CO_2 Permeability Coefficients*

Photosynthesis rates were measured by oxygen evolution except at the lowest $[CO_2]$ where CO_2 assimilation was measured by infrared gas analysis. After determining control rates, at a $[CO_2]$ of 0.035% (v/v) in the supplied air, the water provided to the leaf was supplemented with DCDP (final concentration 4 mM). After inhibition of net photosynthesis the $[CO_2]$ in the air supply was progressively increased and rates measured again. Permeability coefficients were calculated as described in the text.

Species and C_4 Subgroup	Chl[a] Content	CO_2 Concentration	Net Photosynthesis Rate		Permeability Coefficient, P_{CO_2}	
			Control	4 mM DCDP		
	mg m^{-2}	*% (v/v)*	*μmol min^{-1} $(mgChl)^{-1}$*		*μmol min^{-1} $(mgChl)^{-1}$ mM^{-1}*	*cm s^{-1}*[b]
Panicum miliaceum (NAD-ME)	280	0.026[c]	6.4	−0.3		
		1.62		8.1	21	5.6×10^{-3}
		4.90	13.7	11.0		
Amaranthus edulis (NAD-ME)	269	0.019[c]	6.4	−0.2		
		1.62		5.1	12	1.8×10^{-3}
		5.05	12.0[d]	6.1		
Chloris gayana (PCK)	383	0.029[c]	4.6	−0.4		
		1.61		4.2	10	2.7×10^{-3}
		5.00	11.0	9.6		
Urochloa panicoides (PCK)	243	0.030[c]	6.2	−0.7		
		1.71		4.2	9	2.4×10^{-3}
		4.9	15.9	9.3		
Panicum maximum (PCK)	373	0.027[c]	5.9	−0.3		
		1.63		6.1	14	3.8×10^{-3}
		5.20	12.7	11.5		
Echinochloa crusgalli (NADP-ME)	247	0.030[c]	5.0	−0.2		
		1.59		4.0	9	2.4×10^{-3}
		5.10	12.8	5.9		
Digitaria sanguinalis (NADP-ME)	372	0.024[c]	6.3	−0.2		
		1.71		2.7	6	1.6×10^{-3}
		5.21	11.5	3.8		

[a] Chl content is given to allow conversion of photosynthesis rates and P_{CO_2} values to a leaf area basis. [b] Calculated using values of bundle sheath cell surface area per mg leaf Chl determined in Ref. 12. [c] CO_2 concentration in air leaving the leaf chamber for control rates. For DCDP-inhibited leaf sections the leaving $[CO_2]$ was similar to the $[CO_2]$ in the supplied air (0.035% v/v). [d] Measured on another equivalent leaf section.

this study (6–21 μmol min^{-1} [mg Chl]$^{-1}$ mM^{-1} for a range of C_4 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 C_4 species and the highest for NAD-ME-types. From the combined results, average values (μmol min^{-1} [mg Chl]$^{-1}$ mM^{-1}) for each C_4 subgroup are: NADP-ME, 7, PCK, 15, and NAD-ME, 22. These P_{CO_2} 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 CO_2 and NADP-ME-types the least leaky, of the C_4 subgroups (6).

The values obtained for the CO_2 permeability coefficients for bundle sheath cells may also be compared to values obtained for C_3 mesophyll cells. Using various procedures the transfer resistances (essentially the inverse of the permeability coefficient) for C_3 mesophyll cells have been determined to be in the range 1.2 to 2.4 bar m^2 s mol^{-1} (or about 6 s 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 CO_2 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 C_3 mesophyll cells or up to 200 times lower than for lipid bilayers. This reflects the extent of the barrier to diffusion of CO_2 that C_4 bundle sheath cells have evolved to enable them to perform their specialised function in C_4 photosynthesis.

CONCLUDING COMMENTS

Data is presented which shows that, with the C_4 acid cycle specifically inhibited, it is still possible for C_4 leaves to carry out high rates of photosynthesis when provided with a sufficiently high CO_2 concentration. The relatively high concentrations of CO_2 required provide evidence that CO_2 diffusion between mesophyll and bundle sheath cells is highly restricted, as inferred from earlier studies (11). We determined permeability coefficients for CO_2 diffusion into bundle sheath cells which show these cells to be at least 100-fold less permeable to CO_2 than C_3 mesophyll cells. These permeability coefficient values are a critical component of quantitative models developed to describe the steady-state inorganic carbon status of C_4 species (17) and C_4 photosynthesis (*e.g.* ref. 6). The lipid-polymer, suberin, appears the most likely structural component responsible for the CO_2 diffusion barrier (see introduction) though this has yet to be unambigously 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 C_4 species.

The experiments reported here also show that by elevating the CO_2 concentration it may be feasible to rescue C_4 plants which have an ineffective C_4 acid cycle. Hence, a search for mutants with lesions in the C_4 acid cycle could be undertaken. Whether photosynthesis can be maintained in the long-term under these conditions, however, needs to be established.

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