Effects of the Phosphoenolpyruvate Carboxylase Inhibitor 3,3-Dichloro-2-(Dihydroxyphosphinoylmethyl)propenoate on Photosynthesis

C₄ Selectivity and Studies on C₄ Photosynthesis

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ABSTRACT

The effect of 3,3-dichloro-2-(dihydroxyphosphinoylmethyl)propenoate (DCDP), an analog of phosphoenolpyruvate (PEP), on PEP carboxylase activity in crude leaf extracts and on photosynthesis of excised leaves was examined. DCDP is an effective inhibitor of PEP carboxylase from Zea mays or Panicum miliaceum; 50% inhibition was obtained at 70 or 350 micromolar, respectively, in the presence of 1 millimolar PEP and 1 millimolar HCO₃⁻. When fed to leaf sections via the transpiration stream, DCDP at 1 millimolar strongly inhibited photosynthesis in C₄ species (75-98% inhibition for a range of seven C₄ species), but only moderately in C₃ species (12-46% for four C₃ species), suggesting different mechanisms of inhibition for each photosynthetic type. The response of P. miliaceum (C₄) net photosynthesis to intercellular pCO₂ showed that carboxylation efficiency, as well as the CO₂ saturated rate, are lower in the presence of DCDP and supported the view that carboxylation efficiency in C₄ species is directly related to PEP carboxylase activity. A fivefold increase in intercellular pCO₂ over that occurring in P. miliaceum under normal photosynthesis conditions only increased net photosynthesis rate in the presence of 1 millimolar DCDP from zero to about 5% of the maximal uninhibited rate. Therefore, it seems unlikely that direct fixation of atmospheric CO₂ by the bundle sheath cells makes any significant contribution to photosynthetic CO₂ assimilation in C₄ species. The results support the concept that C₄-selective herbicides may be developed based on inhibitors of C₄ pathway reactions.

C₄ plants dominate in lists of the world’s worst weeds (4). Inhibitors of the C₄ pathway should prevent photosynthesis and may have potential as herbicides selective to C₄ weeds (7), but as yet no good C₄-selective inhibitors of photosynthesis have been described. We recently reported that DCDP¹ is a potent, selective inhibitor of PEP carboxylase from leaves of both C₄ and C₃ plants (11). In examining the feasibility that C₄-selective herbicides may be designed based on inhibitors of the C₄ pathway enzymes, it was of interest to test this compound on photosynthesis in C₃ and C₄ species.

A specific inhibitor of the C₄ pathway may also be useful in studies on other aspects of C₄ photosynthesis, particularly the CE, and the CO₂ diffusibility of the bundle sheath-mesophyll interface of C₄ plants. C₄ plants reach maximum rates of photosynthesis at lower intercellular [CO₂] than C₃ plants, and have higher CE (i.e. carbon gain per unit increase in [CO₂] at subsaturating [CO₂]) determined from the slope of the assimilation response to intercellular [CO₂]. These gas-exchange features have been attributed to the operation of the C₄ pathway concentrating CO₂ in bundle sheath cells at the site of Rubisco, thereby preventing O₂ inhibition of carboxylation, and to the involvement of the different primary carboxylases in the C₄ and C₃ photosynthetic types (4, 8). In normal air and at high light intensity CE in C₃ plants is correlated with the amount of Rubisco in the leaf (18). This has been demonstrated by varying the amount of Rubisco by differing levels of nitrogen nutrition or light intensity during growth (18). In contrast, in C₃ species CE has been related to the high maximum velocity of PEP carboxylase (generally severalfold higher than maximum photosynthesis rate) and its high effective affinity for the inorganic carbon substrate (while HCO₃⁻ is the substrate for PEP carboxylase, in C₄ mesophyll cells in the presence of carbonic anhydrase HCO₃⁻ would be in rapid equilibrium with a relatively low concentration of CO₂). Thus, the carboxylation rate at low [CO₂] can be high relative to maximum photosynthesis capacity. This involvement of PEP carboxylase in CE is not easily demonstrated experimentally, however, as it is difficult to manipulate C₄ pathway activity while keeping constant the activity of the C₄ photosynthetic carbon reduction cycle (see, e.g. Refs. 16 and 17).

A critical feature for the effective functioning of the C₄ pathway is that the interface between the bundle sheath and mesophyll cells in C₄ species has a low diffusibility to CO₂ to allow generation of high CO₂ concentrations in bundle sheath cells. The rate of CO₂ leakage from bundle sheath cells is unknown, though it is probably less than 20% of the photosynthesis rate (6). The extent to which this CO₂ barrier restricts

¹ Abbreviations: DCDP, 3,3-dichloro-2-(dihydroxyphosphinoylmethyl)propenoate; PEP, phosphoenolpyruvate; CE, carboxylation efficiency; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; G6P, glucose 6-phosphate; RuBP, ribulose 1,5-bisphosphate; pCO₂, partial carbon dioxide pressure.
entry and direct fixation of atmospheric CO₂ by Rubisco in bundle sheath cells under normal conditions is uncertain; although short-term ¹⁴CO₂-labeling experiments have shown 100% fixation into C₄ acids when extrapolated to zero time, indicating no direct fixation by Rubisco (7), it has been suggested elsewhere, based on experiments with leaf slices, that 10 to 15% of carbon assimilation may be due to direct fixation in bundle sheath cells (2, 14, 15). As well as under normal conditions of photosynthesis, it is also of interest to know in relation to the feasibility of C₄-specific herbicides whether C₃ plants can continue to assimilate atmospheric CO₂ in the absence of a functional C₄ pathway, by direct fixation of CO₂ in the bundle sheath cells.

In this report it is shown that DCDP is an effective inhibitor of PEP carboxylase in crude leaf extracts under various conditions, and of photosynthesis with some degree of selectivity toward C₄ species. The responses of photosynthesis in a C₄ species to variations in intercellular [CO₂] and incident light intensity in the presence of this inhibitor have also been examined.

MATERIALS AND METHODS

Chemicals

Biochemicals and reagent enzymes were obtained from Sigma Chemical Co. or Boehringer Mannheim. DCDP was synthesized at CSIRO (11) and kindly provided by Dr. R. L. N. Harris and Ms. H. G. McFadden. For some preparations which were isolated as the monocyclohexylammonium salt, the free acid was obtained by treatment on a small column of cation-exchange resin (Dowex-50) in the H⁺ form, and neutralized with dilute KOH.

Plant Material

Seedlings were grown in a glasshouse in sterile soil under natural illumination with, for most species, a temperature maintained between 20 and 30°C. For Pisum sativum and Brassica napus growth temperature was between 18 and 26°C. The youngest, fully expanded leaves of plants between 2 and 4 weeks old were cut, then immediately recut carefully under water before use. Usually the top of the leaf was also cut off leaving a leaf section between 10 and 13 cm long.

Preparation of Crude Leaf Extracts and PEP Carboxylase Assays

Before extraction leaves were illuminated for approximately 1 h at 1000 μmol quanta m⁻²s⁻¹ provided by a Hg-vapor lamp (Phillips HPL), or, for some experiments as indicated in "Results," darkened overnight. For extraction, leaf sections (approximately 0.2 g fresh weight) were quickly debined then homogenized for 40 s in a mortar and pestle with 1 mL of grinding medium containing 50 mM Hepes-KOH (pH 7.2), 10 mM MgCl₂, 1 mM DTT, 25% (v/v) glycerol, and 2% (w/v) BSA (3). Then an additional 0.5 mL of grinding medium was added and the mixture ground for a further 40 s. After removing 0.1-mL portions for Chl determination, the homogenate was transferred to an Eppendorf tube and centrifuged at 10,000g for 30 s. The supernatant was kept at 25°C and used as the source of PEP carboxylase. Enzyme activity was assayed spectrophotometrically at 25°C by the decrease in A₃₄₀ due to oxidation of NADH in the presence of excess malate dehydrogenase as coupling enzyme. The assay mixture contained 25 mM Hepes-KOH (pH 7.5), 10 mM MgCl₂, 1 mM NaHCO₃, 0.2 mM NADH, 2 IU of malate dehydrogenase, and various concentrations of PEP, DCDP, and other metabolites as indicated in "Results."

Photosynthesis Measurements

Net photosynthesis was measured in an open gas-exchange system using a clamp-on leaf chamber (PLC(B)) and infrared gas analyzer (LCA-2) (Analytical Development Co., Ltd.). Usually a leaf section with the cut base in water was clamped so that the top 2.5 cm of leaf was in the chamber and photosynthesis of this section was monitored continuously. Illumination (routinely 1000 μmol quanta m⁻²s⁻¹) was provided by an incandescent lamp using a glass dish of water between the lamp and the chamber 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. Relative humidity and air temperature were measured by the built-in chamber sensors and leaf temperature by a differential thermocouple system. Air was supplied at 200 or 400 mL min⁻¹ from tanks containing 350 or 360 μL CO₂/L, or by mixing CO₂-free air and 0.1% CO₂ to obtain the desired concentration. To examine the effect of DCDP on net photosynthesis, leaf sections were illuminated until a steady (control) rate of photosynthesis was attained, then DCDP solution added to the feed solution to give the required concentration and photosynthesis followed with a chart recorder. Further details for individual experiments are given in "Results." Intercellular pCO₂ was calculated according to Von Caemmerer and Farquhar (18). Leaf absorbance (400–700 nm) was determined using a Varian 634 spectrophotometer fitted with an integrating sphere attachment.

Photosynthetic Oxygen Evolution by Isolated Bundle Sheath Cells

The methods for preparation of bundle sheath strands and measurement of light- and HCO₃⁻-dependent photosynthetic O₂ evolution were based on published methods (1).

Chl

Chl was determined in 90% acetone extracts according to the equations of Jeffrey and Humphrey (10).

RESULTS

Effects of DCDP on PEP Carboxylase Activity in Crude Leaf Extracts

Previous studies on the mechanism of DCDP inhibition were performed mainly with partially purified PEP carboxylase at pH 8.0 (11). In order to examine effects of the inhibitor under conditions which might better approximate the physiological situation, inhibition was measured at pH 7.5 using
crude extracts from illuminated leaves as a source of enzyme. The results (Fig. 1) show that DCDP is effective against maize PEP carboxylase under these conditions giving 50% inhibition at 35 μM and about 80% inhibition at 200 μM in the presence of 0.4 mM PEP and saturating (1 mM) HCO₃⁻. Like the response of isolated enzyme at pH 8.0 (11), the inhibition of the maize enzyme measured here was decreased by the presence of higher PEP concentrations, indicating a degree of competition between PEP and DCDP. With the partially purified maize leaf enzyme, DCDP is a linear competitive inhibitor with respect to PEP, with a Kᵢ-value of 80 μM at pH 8 and 5 mM Mg²⁺ (11). In contrast, inhibition of crude PEP carboxylase from P. miliaceum leaves was essentially not affected by PEP concentration from 0.4 to 5 mM suggesting some difference in the mechanisms of reaction or inhibition between these enzymes. Although DCDP was less potent on the enzyme from P. miliaceum than maize it was still an effective inhibitor giving about 80% inhibition at 2 mM in the presence of 5 mM PEP.

Maize leaf PEP carboxylase activity may be regulated by various metabolites and illumination or darkening of leaves (3, 9). Inhibition of PEP carboxylase by DCDP was measured in the presence of G6P, as a representative activator, and malate, a possible physiological inhibitor, using freshly prepared extracts from both illuminated and darkened leaves (Table I). In the absence of DCDP, activity with 1 mM PEP alone at pH 7.5 was similar in light or dark extracts, and in each case activity was approximately doubled by 5 mM G6P. In some experiments activity was higher in illuminated leaf extracts, particularly at lower PEP concentrations (not shown). Malate was considerably more effective as an inhibitor of enzyme from darkened leaves, either in the absence or presence of G6P. These results are similar to those reported elsewhere (3, 9) and indicate the various enzyme forms or enzyme-metabolite complexes that can occur. DCDP at 50 μM inhibited activity in the light extract about 30% under all conditions and by 40 to 60% at 200 μM DCDP. With enzyme from darkened leaves the extent of inhibition by DCDP was more variable. There was little further inhibition when the enzyme was already inhibited by malate (in the absence or presence of G6P), while DCDP was about as effective as on the light enzyme in the presence of PEP alone, or PEP plus G6P. Overall, the results suggest that the enzyme retains sensitivity to DCDP under a variety of conditions, except where it is already substantially inhibited by malate in extracts of darkened leaves.

Effects of DCDP on Net Photosynthesis in C₃ and C₄ Species

The effects of DCDP on photosynthesis were examined using excised leaves or leaf sections held in a clamp-on leaf chamber which allowed continuous monitoring of changes in photosynthesis, transpiration, and leaf temperature following administration of DCDP via the transpiration stream. Under the experimental conditions employed, once steady-state photosynthesis was established on illumination (30–60 min), control leaves usually maintained the same rate, or sometimes showed a steady slow decline (about 5% per h), over at least 4 h. Typical results for the effect of feeding DCDP to C₃ and C₄ leaves carrying out steady-state photosynthesis in normal air are shown in Figure 2. With C₃ species, after a very short lag period (about 2 min) there was a rapid, substantial decline in CO₂ fixation. The net photosynthesis rate was decreased by approximately 80% within 40 min following the start of feeding 1 mM DCDP for the C₃ species shown, then declined more slowly to less than 10% of the uninhibited rate. The rate of decline of photosynthesis was dependent on the inhibitor concentration; at lower concentrations the initial lag was longer and several hours were required to achieve the same degree of inhibition (not shown). In contrast, for C₄ species photosynthesis was only moderately inhibited (Fig. 2). After a longer lag period of about 5 min photosynthesis declined to a rate of about 60% or more of the original rate which then remained almost constant.

Table I. Effect of DCDP on PEP Carboxylase Activity in Crude Extracts of Illuminated or Darkened Z. mays Leaves in the Absence or Presence of Malate and G6P

<table>
<thead>
<tr>
<th>Metabolite Additions</th>
<th>PEP Carboxylase Activity*</th>
<th>Control</th>
<th>+50 μM</th>
<th>+200 μM</th>
<th>DCDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Illuminated leaf</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEP</td>
<td>4.7</td>
<td>3.2 (68)</td>
<td>1.8 (38)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEP, malate</td>
<td>2.0</td>
<td>1.4 (70)</td>
<td>0.9 (40)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEP, G6P</td>
<td>9.2</td>
<td>6.5 (71)</td>
<td>4.4 (48)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEP, malate, G6P</td>
<td>4.9</td>
<td>3.3 (67)</td>
<td>2.9 (59)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Darkened leaf</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEP</td>
<td>5.4</td>
<td>2.9 (54)</td>
<td>1.9 (35)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEP, malate</td>
<td>0.6</td>
<td>0.6 (100)</td>
<td>0.5 (83)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEP, G6P</td>
<td>10.6</td>
<td>7.3 (69)</td>
<td>5.5 (52)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEP, malate, G6P</td>
<td>3.2</td>
<td>2.2 (69)</td>
<td>2.3 (72)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Crude leaf extracts were prepared and enzyme activity measured as described in "Materials and Methods." Concentrations of added metabolites were 1.0 mM PEP, 5 mM malate, and 5 mM G6P. Figures in parentheses indicate the percentage of control activities in the absence of DCDP.
The results of these and other experiments where a range of plant species were examined for DCDP inhibition of net photosynthesis are summarized in Table II. The C₄ species were selected to represent each of the three subgroups of C₄ species, defined by decarboxylation mechanism, in addition to C₃ species. Photosynthesis of all C₄ leaves was substantially inhibited by DCDP; after 1 h of feeding 1 mm DCDP, inhibition ranged from 79 to 98%. Photosynthesis in C₃ species was less sensitive to DCDP, with inhibition of between 12 and 46%. It is unlikely that the differences in the effects of DCDP on net photosynthesis in C₃ and C₄ leaves were due to differences in inhibitor uptake since both photosynthetic types had substantial rates of transpiration under the experimental conditions. These results clearly demonstrate that DCDP has preferential effects depending whether photosynthesis occurs via the C₃ or C₄ mechanism, with C₄ species being inhibited to a much greater extent. The results suggest that a major effect of DCDP is on the C₄ pathway and at a point which is common to all C₄ subgroups.

Since transpiration was decreased to a much lesser extent than CO₂ fixation, being only inhibited by about 40% in C₄ leaves when fixation was inhibited by 90% (not shown), it is possible that there may be some accumulation of inhibitor in the leaf, perhaps leading to relatively high inhibitor concentrations compared to the feed solution. Despite this possibility, for several C₄ species when the DCDP feed solution was replaced by water, there was a rapid recovery of photosynthesis to within a few percent of their initial rates (Fig. 2). Transpiration rates also returned to original values (not shown). This indicates that DCDP must undergo rapid removal from a specific site of action, either by sequestration or metabolism to some ineffective product. The mechanism of this apparent removal has not been further investigated. These results indicate that inhibition of photosynthesis in C₄ species was not due to some nonspecific toxicity or irreversible damage to the photosynthetic apparatus.

No recovery of photosynthesis in C₃ species was obtained on replacement of the DCDP feed solution with water (not shown), providing further evidence that the mechanism of inhibition of photosynthesis is different in C₃ and C₄ leaves. Since PEP carboxylase is involved in stomatal opening (19) there was a possibility that DCDP inhibition in C₃ species may be due to a decreased intercellular pCO₂ caused by stomatal limitation to CO₂ diffusion. To test this, DCDP inhibition of photosynthesis was measured in C₃ leaves exposed to air containing a saturating concentration of CO₂ (0.1%) which should overcome stomatal limitations. These experiments (Table III) involved measuring control photosynthesis rates at high and normal pCO₂, then measuring rates again at each pCO₂ following a period of DCDP treatment at normal pCO₂. The results indicate that exposure to high pCO₂ after DCDP treatment caused a recovery of photosynthesis rate from 66 to 79% of the respective control rates in barley, and from 59 to 79% in wheat. This partial recovery suggests that a portion of the DCDP inhibition of photosynthesis in C₃ species may be due to stomatal effects. Consistent with this, in other experiments feeding 1 mM DCDP to wheat leaves continuously exposed to air containing 0.1% CO₂ caused only about 10% inhibition of photosynthesis compared to controls (not shown).

A possible effect of DCDP on reactions of the C₃ photosynthetic carbon reduction cycle in C₄ species was also tested using isolated bundle sheath cells. Bicarbonate and light dependent photosynthetic oxygen evolution by bundle sheath
concentrations of DCDP to leaf sections via the transpiration stream and measuring photosynthesis using a leaf chamber. After 1 to 2 h, the rate of photosynthesis was decreased at atmospheric CO₂ concentration (340 μL/L) by the amount shown (indicated by the rates at atmospheric CO₂ concentration shown by the arrows in Fig. 3). At this point the CO₂ concentration in the supplied air was increased to approximately 1012 μL/L and photosynthesis measurements at the various CO₂ concentrations begun. The high CO₂ concentrations in the air stream caused a rapid closing of stomata (stomatal conductance decreased from 293 to 118 mmol m⁻²s⁻¹ in the control), effectively slowing further rapid uptake of inhibitor, and keeping the extent of inhibition constant within reasonable limits over the period of the experiment. The control leaf showed a high CE (determined by the slope of the response at low intercellular pCO₂) and reached a maximum at external CO₂ concentrations a little above atmospheric. Following treatment with increasing concentrations of DCDP, both the CE and the maximum photosynthesis rate attained were progressively decreased. Also, the pCO₂ at which photosynthesis became saturated was increased. With treatments at high DCDP concentrations, when photosynthesis was markedly inhibited, there were small but distinguishable increases in photosynthesis rate at higher pCO₂. For example, after treatment with 1 mM DCDP, net photosynthesis was zero at a pCO₂ corresponding to atmospheric CO₂ concentration but was increased to about 1 μmol m⁻²s⁻¹ (about 3% of maximum) at a pCO₂ of about 900 μbar, some fivefold higher than the pCO₂ which occurs in uninhibited leaves in normal air. It was not possible to measure photosynthesis rates at still higher pCO₂ due to equipment limitations. As a result of DCDP treatment, the apparent CO₂ compensation point of P. miliaceum was greatly increased from approximately 5 μbar to about 240 μbar (with 1 mM DCDP treatment). The negative rate in CO₂-free air, which presumably reflects leaf respiration, was not markedly affected by inhibitor treatment. Photosynthesis in the control leaf and leaves treated with low concentrations of DCDP was slightly inhibited by high pCO₂; this effect was not further investigated.

The effect of incident light intensity on photosynthesis at near atmospheric CO₂ concentrations was examined (Fig. 4). In this case, photosynthesis measurements were made on a P. miliaceum leaf section at various light intensities and then the water supplied to the leaf was replaced by 0.25 mM DCDP, which caused a gradual decline in photosynthesis. When steady inhibition of photosynthesis in the leaf was attained, measurements at a range of intensities were again conducted. The figure shows that DCDP lowers both the maximum rate of photosynthesis at light saturation and the incident light intensity at which saturation occurred. However, there was no effect on the efficiency of utilizing low light intensities determined from the initial slope of the light-response curves. Using an absorbance figure of 0.7, determined for the same leaf after the experiment, a quantum yield of 0.04 μmol CO₂ fixed per μmol photon absorbed was calculated for the leaf both before and after inhibitor treatment. In leaves where carbon fixation is limited and photoinhibition occurs, the

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Barley Photosynthesis Rate (μmol m⁻²s⁻¹)</th>
<th>Barley Percent of control</th>
<th>Wheat Photosynthesis Rate (μmol m⁻²s⁻¹)</th>
<th>Wheat Percent of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.5</td>
<td>100</td>
<td>20.7</td>
<td>100</td>
</tr>
<tr>
<td>High CO₂</td>
<td>15.8</td>
<td>100</td>
<td>14.5</td>
<td>100</td>
</tr>
<tr>
<td>Low CO₂</td>
<td>10.5</td>
<td>66</td>
<td>8.5</td>
<td>59</td>
</tr>
<tr>
<td>Treated (1 mM DCDP) Low CO₂</td>
<td>18.7</td>
<td>79</td>
<td>16.3</td>
<td>79</td>
</tr>
<tr>
<td>High CO₂</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Table III. Inhibition of Net Photosynthesis in Barley and Wheat Leaf Sections by DCDP at Normal and High pCO₂ Levels

Figure 3. Response of photosynthesis rate of P. miliaceum leaf sections to intercellular partial pressure of CO₂ following treatment with various concentrations of DCDP. Leaf sections, with their cut bases in water, were illuminated (approximately 1000 μE m⁻²s⁻¹) and net photosynthesis measured with a clamp-on leaf chamber. For each section, after a period of equilibration at [CO₂] of 260 μL/L, DCDP (final concentration as shown) was supplied to the cut base. After a further period of gradual inhibition of photosynthesis rate, the CO₂ in the supplied air was changed to 1012 μL/L and then decreased in steps, and steady-state photosynthesis rates measured. Arrows indicate intercellular pCO₂ corresponding to atmospheric [CO₂] (340 μL/L). Uninhibited rates were similar for all leaf sections. The temperature in the chamber was 27.2 ± 1°C.

Effects of DCDP on Photosynthetic Response to CO₂ Concentration and Incident Light Intensity in P. miliaceum.

The response of photosynthesis of P. miliaceum leaf sections to the intercellular partial pressure of CO₂ after inhibition with DCDP is shown in Figure 3. In these experiments the extent of inhibition was varied by feeding a range of concentrations of DCDP to leaf sections via the transpiration stream and measuring photosynthesis using a leaf chamber. After 1 to 2 h, the rate of photosynthesis was decreased at atmospheric CO₂ concentration (340 μL/L) by the amount shown (indicated by the rates at atmospheric CO₂ concentration shown by the arrows in Fig. 3). At this point the CO₂ concentration in the supplied air was increased to approximately 1012 μL/L and photosynthesis measurements at the various CO₂ concentrations begun. The high CO₂ concentrations in the air stream caused a rapid closing of stomata (stomatal conductance decreased from 293 to 118 mmol m⁻²s⁻¹ in the control), effectively slowing further rapid uptake of inhibitor, and keeping the extent of inhibition constant within reasonable limits over the period of the experiment. The control leaf showed a high CE (determined by the slope of the response at low intercellular pCO₂) and reached a maximum at external CO₂ concentrations a little above atmospheric. Following treatment with increasing concentrations of DCDP, both the CE and the maximum photosynthesis rate attained were progressively decreased. Also, the pCO₂ at which photosynthesis became saturated was increased. With treatments at high DCDP concentrations, when photosynthesis was markedly inhibited, there were small but distinguishable increases in photosynthesis rate at higher pCO₂. For example, after treatment with 1 mM DCDP, net photosynthesis was zero at a pCO₂ corresponding to atmospheric CO₂ concentration but was increased to about 1 μmol m⁻²s⁻¹ (about 3% of maximum) at a pCO₂ of about 900 μbar, some fivefold higher than the pCO₂ which occurs in uninhibited leaves in normal air. It was not possible to measure photosynthesis rates at still higher pCO₂ due to equipment limitations. As a result of DCDP treatment, the apparent CO₂ compensation point of P. miliaceum was greatly increased from approximately 5 μbar to about 240 μbar (with 1 mM DCDP treatment). The negative rate in CO₂-free air, which presumably reflects leaf respiration, was not markedly affected by inhibitor treatment. Photosynthesis in the control leaf and leaves treated with low concentrations of DCDP was slightly inhibited by high pCO₂; this effect was not further investigated.

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Figure 4. Effect of incident light intensity on photosynthesis rate in a P. miliaceum leaf initially and following feeding with DCDP. After a period of equilibration at approximately 1200 μE m⁻² s⁻¹, photosynthesis rates of an excised leaf, with its cut base in water or DCDP solution, were measured over a range of light intensities (highest first) using a clamp-on leaf chamber as described in "Materials and Methods." Rates were measured at various incident light intensities after feeding water (O), and after subsequent feeding with 0.25 mm DCDP for approximately 70 min (△), and 180 min (□). The temperature in the chamber decreased from 25.3 to 22.0°C (O), and 27.5 to 23.5°C (△,□) as light intensity decreased; the [CO₂] varied between 325 and 360 μL/L.

The results presented here and earlier (11) indicate that DCDP is a reasonably potent, selective inhibitor of maize leaf PEP carboxylase and is competitive with respect to PEP. While the mechanism is uncertain, the compound was also effective on enzyme from P. miliaceum and has been shown previously to inhibit PEP carboxylase from a range of both C₃ and C₄ species, but not other PEP-utilizing enzymes (11). At least for maize, the various forms of the enzyme from illuminated or darkened leaves in the presence or absence of regulatory metabolites were all sensitive to DCDP, except where activity was already substantially inhibited in darkened-leaf extracts in the presence of malate. The compound is, thus, a useful tool for studies on C₄ photosynthesis or other metabolic processes involving PEP carboxylase.

DCDP is an inhibitor of net photosynthesis in leaves when fed via the transpiration stream and shows a degree of selectivity between C₃ and C₄ species. While C₃ species were rapidly and sometimes completely inhibited by the compound, C₄ species were only partially inhibited. It is most likely that inhibition of photosynthesis in C₃ species occurs as a result of inhibition of PEP carboxylase. By decreasing the primary carboxylation reaction DCDP presumably limits the operation of the C₄ pathway and effectively prevents the supply of CO₂ to the bundle sheath cells. Since the PEP carboxylase reaction is common to the three C₃ subgroups, all C₄ plants should be affected, consistent with observations made here. While secondary effects of DCDP cannot be ruled out unequivocally, the lack of effects of the compound on other PEP-utilizing enzymes (11), other C₄ pathway enzymes (CLD Jenkins, unpublished observations), or photosynthesis by isolated bundle sheath strands suggests a specific effect on PEP carboxylase. The lack of effect of DCDP on the quantum yield of net photosynthesis, and the recovery of photosynthesis following removal of DCDP from the feed solution, provide evidence against other nonselective toxic effects, though the mechanism for removal of inhibitor from the leaf remains uncertain.

The reason for the partial inhibition of photosynthesis by DCDP in C₃ species is less clear. Since inhibition usually was observed after a long lag period, some nonspecific toxicity may occur as the inhibitor accumulates to higher concentrations in the leaf. Alternatively, the effect may again be due to inhibition of C₃ PEP carboxylase (11). Although many possible functions have been suggested for PEP carboxylase in C₃ leaves a role in anaplerotic metabolism has been clearly shown (12) but it is not clear how inhibition of this function would affect net photosynthesis in the short term. However, PEP carboxylase has also been implicated in stomatal functioning (19) and interference with this mechanism by DCDP could affect photosynthesis by limiting CO₂ diffusion into the leaf. Consistent with this view, in the present work a partial recovery of photosynthesis was achieved by increasing the CO₂ concentration in the supplied air. In C₄ plants this explanation does not seem likely since, for P. miliaceum, assessment of stomatal limitation to photosynthesis based on the CO₂ response curves according to Farquhar and Sharkey (5) indicated that stomatal limitation to photosynthesis did not increase following treatment with DCDP. It is also evident from Figure 3 that in the presence of DCDP the intercellular pCO₂, at atmospheric CO₂ concentration, in a C₄ species was not decreased.

The results of Figure 3 provide a useful demonstration that CE in C₄ plants is dependent on PEP carboxylase activity, in contrast to C₃ plants where it is correlated with the amount of Rubisco in leaves (18). In the presence of increasing concentrations of inhibitor the activity of PEP carboxylase in P. miliaceum leaves would be progressively decreased, and this results in a progressive decrease in CE although the amount of Rubisco remains constant. Lowering of CE could be because of effects of DCDP on either the apparent Kₘ for HCO₃⁻ of PEP carboxylase or on the apparent Vₘₐₓ at saturating HCO₃⁻ concentration. While detailed studies on the effects of DCDP on PEP carboxylase kinetics with respect to HCO₃⁻ have yet to be conducted, the virtual lack of recovery of photosynthesis at high pCO₂ in the presence of DCDP argues the latter case.

The photosynthesis rate in C₄ plants is probably dependent on the RuBP regeneration rate (for which the capacity would remain the same for all treatments in Figure 3), the amount of Rubisco, and the capacity of the C₄ pathway to supply CO₂ to the bundle sheath cells and generate a high intracellular CO₂ concentration. This capacity is given by the maximum rate which the C₄ cycle can achieve less the rate of inorganic carbon leakage from bundle sheath cells. Presumably, it is this C₄ pathway capacity that is limiting photosynthesis rates in the presence of DCDP. It seems most likely that the maximum rate at saturating CO₂ concentration is depressed because of the lowered rate of supply of CO₂ to the bundle...
sheath cells and the subsequent effect of the lowering of CO₂ concentration in bundle sheath cells on Rubisco activity. The importance of the concentration of CO₂ in bundle sheath cells has been shown by Furubank and Hatch (6) who found a good correlation between the inorganic carbon pool size in C₄ leaves and the photosynthesis rate under various conditions of illumination and CO₂ concentration. Furthermore, a lowered CO₂ concentration in bundle sheath cells may also change the activation state of Rubisco, which could be a contributing factor to the lowered maximum photosynthesis rates. Since quantum yield was unaffected by DCDP treatment, at least at intermediate levels of inhibition of photosynthesis, it appears that there was still a sufficiently high CO₂ concentration in the bundle sheath cells to prevent Rubisco oxygenase activity and the associated photosynthetic CO₂ loss.

DCDP reduced net photosynthesis in C₄ plants to zero at atmospheric CO₂ concentration (Fig. 3). Any remaining photosynthetic carbon fixation was apparently offset by respiratory carbon loss. This zero net fixation at atmospheric CO₂ concentration was increased to about 5% of the maximum uninhibited photosynthesis rate by increasing the intercellular pCO₂ approximately fivefold. It is possible that the increase may be due to direct fixation of atmospheric CO₂ by Rubisco in bundle sheath cells rather than via the normal C₃ pathway. If this is the maximal rate of photosynthesis that can be attained by direct fixation at a fivefold elevated pCO₂ in the absence of the C₄ pathway, it seems unlikely that direct bundle sheath fixation can account for much net assimilation in normal air in the presence of a fully active, normally functioning C₄ pathway. This would be consistent with the view of Hatch (7) but not with other workers (2, 14). It appears that in the evolutionary development of the C₄ pathway, by gaining a permeability barrier to CO₂ between mesophyll and bundle sheath cells, C₄ species have virtually lost the capability to fix atmospheric CO₂ via the usual C₃ mechanism.

The results presented here support the concept that C₄-selective herbicides may be developed based on inhibitors of C₄ pathway reactions. The PEP carboxylase inhibitor used here, when fed to leaves, decreased photosynthesis virtually to zero in C₄ species, but had lesser effects on C₃ leaves, at least in the short term. Irreversible inhibitors of PEP carboxylase, or other C₄ pathway enzymes, that could penetrate leaves would be of interest to test as potential C₄-selective herbicides.

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LITERATURE CITED


