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Effects of the Phospho*enol*pyruvate Carboxylase Inhibitor 3,3-Dichloro-2-(Dihydroxyphosphinoylmethyl)propenoate on Photosynthesis

C_4 Selectivity and Studies on C_4 Photosynthesis

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ABSTRACT

The effect of 3,3-dichloro-2-(dihydroxyphosphinoylmethyl)-propenoate (DCDP), an analog of phospho*enol*pyruvate (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 HCO_3^-. When fed to leaf sections via the transpiration stream, DCDP at 1 millimolar strongly inhibited photosynthesis in C_4 species (79–98% inhibition for a range of seven C_4 species), but only moderately in C_3 species (12–46% for four C_3 species), suggesting different mechanisms of inhibition for each photosynthetic type. The response of *P. miliaceum* (C_4) net photosynthesis to intercellular pCO_2 showed that carboxylation efficiency, as well as the CO_2 saturated rate, are lowered in the presence of DCDP and supported the view that carboxylation efficiency in C_4 species is directly related to PEP carboxylase activity. A fivefold increase in intercellular pCO_2 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 CO_2 by the bundle sheath cells makes any significant contribution to photosynthetic CO_2 assimilation in C_4 species. The results support the concept that C_4-selective herbicides may be developed based on inhibitors of C_4 pathway reactions.

C_4 plants dominate in lists of the world's worst weeds (4). Inhibitors of the C_4 pathway should prevent photosynthesis and may have potential as herbicides selective to C_4 weeds (7), but as yet no good C_4-selective inhibitors of photosynthesis have been described. We recently reported that DCDP[1] is a potent, selective inhibitor of PEP carboxylase from leaves of both C_4 and C_3 plants (11). In examining the feasibility that C_4-selective herbicides may be designed based on inhibitors of the C_4 pathway enzymes, it was of interest to test this compound on photosynthesis in C_3 and C_4 species.

A specific inhibitor of the C_4 pathway may also be useful in studies on other aspects of C_4 photosynthesis, particularly the CE, and the CO_2 diffusibility of the bundle sheath-mesophyll interface of C_4 plants. C_4 plants reach maximum rates of photosynthesis at lower intercellular $[CO_2]$ than C_3 plants, and have higher CE (*i.e.* carbon gain per unit increase in $[CO_2]$ at subsaturating $[CO_2]$, determined from the slope of the assimilation response to intercellular $[CO_2]$). These gas-exchange features have been attributed to the operation of the C_4 pathway concentrating CO_2 in bundle sheath cells at the site of Rubisco, thereby preventing O_2 inhibition of carboxylation, and to the involvement of the different primary carboxylases in the C_4 and C_3 photosynthetic types (4, 8). In normal air and at high light intensity CE in C_3 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 C_4 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 HCO_3^- is the substrate for PEP carboxylase, in C_4 mesophyll cells in the presence of carbonic anhydrase HCO_3^- would be in rapid equilibrium with a relatively low concentration of CO_2). Thus, the carboxylation rate at low $[CO_2]$ 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 C_4 pathway activity while keeping constant the activity of the C_3 photosynthetic carbon reduction cycle (see, *e.g.* Refs. 16 and 17).

A critical feature for the effective functioning of the C_4 pathway is that the interface between the bundle sheath and mesophyll cells in C_4 species has a low diffusibility to CO_2 to allow generation of high CO_2 concentrations in bundle sheath cells. The rate of CO_2 leakage from bundle sheath cells is unknown, though it is probably less than 20% of the photosynthesis rate (6). The extent to which this CO_2 barrier restricts

[1] Abbreviations: DCDP, 3,3-dichloro-2-(dihydroxyphosphinoylmethyl)propenoate; PEP, phospho*enol*pyruvate; CE, carboxylation efficiency; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; G6P, glucose 6-phosphate; RuBP, ribulose 1,5-bisphosphate; pCO_2, partial carbon dioxide pressure.

entry and direct fixation of atmospheric CO_2 by Rubisco in bundle sheath cells under normal conditions is uncertain; although short-term $^{14}CO_2$-labeling experiments have shown 100% fixation into C_4 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 C_4-specific herbicides whether C_4 plants can continue to assimilate atmospheric CO_2 in the absence of a functional C_4 pathway, by direct fixation of CO_2 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 C_4 species. The responses of photosynthesis in a C_4 species to variations in intercellular [CO_2] 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 $m^{-2}s^{-1}$ 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 deribbed 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 $MgCl_2$, 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 10,000*g* 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 A_{340} 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 $MgCl_2$, 1 mM $NaHCO_3$, 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 $m^{-2}s^{-1}$) 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 CO_2/L, or by mixing CO_2-free air and 0.1% CO_2 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 pCO_2 was calculated according to Von Caemmerer and Farquhar (18). Leaf absorptance (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 HCO_3^--dependent photosynthetic O_2 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) HCO_3^-. 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 K_i-value of 80 μM at pH 8 and 5 mM Mg^{2+} (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.

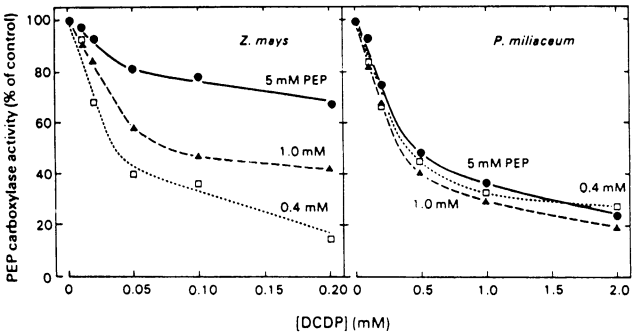

Figure 1. Inhibition by DCDP of PEP carboxylase activity in crude leaf extracts of *Z. mays* and *P. miliaceum*. PEP carboxylase activity in leaf extracts was measured as described in "Materials and Methods" in the presence of varying concentrations of DCDP and PEP as indicated. Maximum activities with 0.4, 1.0, and 5 mM PEP were 3.4, 4.5, and 7.4 μmol min^{-1} (mg Chl)$^{-1}$ for *Z. mays* and 1.9, 4.7, and 16.1 μmol min^{-1} (mg Chl)$^{-1}$ for *P. miliaceum*, respectively. (Note 10-fold difference in scales used for DCDP concentration).

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*

Metabolite Additions	PEP Carboxylase Activity[a]		
	Control	+50 μM DCDP	+200 μM DCDP
	μmol min^{-1} mg Chl^{-1}		
Illuminated leaf			
PEP	4.7	3.2 (68)[b]	1.8 (38)
PEP, malate	2.0	1.4 (70)	0.8 (40)
PEP, G6P	9.2	6.5 (71)	4.4 (48)
PEP, malate, G6P	4.9	3.3 (67)	2.9 (59)
Darkened leaf			
PEP	5.4	2.9 (54)	1.9 (35)
PEP, malate	0.6	0.6 (100)	0.5 (83)
PEP, G6P	10.6	7.3 (69)	5.5 (52)
PEP, malate, G6P	3.2	2.2 (69)	2.3 (72)

[a] 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. [b] Figures in parentheses indicate the percentage of control activities in the absence of DCDP.

Effects of DCDP on Net Photosynthesis in C_3 and C_4 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 C_3 and C_4 leaves carrying out steady-state photosynthesis in normal air are shown in Figure 2. With C_4 species, after a very short lag period (about 2 min) there was a rapid, substantial decline in CO_2 fixation. The net photosynthesis rate was decreased by approximately 80% within 40 min following the start of feeding 1 mM DCDP for the C_4 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 C_3 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.

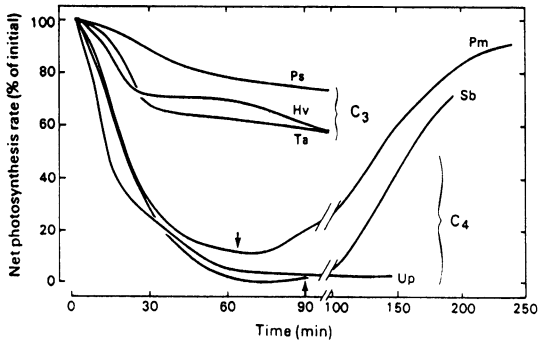

Figure 2. Effect of feeding DCDP to leaf sections of C_3 and C_4 species on net photosynthesis rates. Leaf sections with their cut bases in water were illuminated (approximately 1000 μE $m^{-2}s^{-1}$) and photosynthesis rates measured using a clamp-on leaf chamber as described in "Materials and Methods." Following an equilibration period to attain steady-state photosynthesis, DCDP was added to the feed water (final concentration 1 mM) and rates continuously measured. C_3 species: Ps, *P. sativum*; Hv, *H. vulgare*; Ta, *T. aestivum*; C_4 species: Pm, *P. miliaceum*; Sb, *S. bicolor*; Up, *U. panicoides*. At the times shown by the arrows the inhibitor solution was replaced with water for *P. miliaceum* and *S. bicolor*. Initial photosynthesis rates and other details are given in Table II.

Table II. *Net Photosynthesis Rates of Leaf Sections of a Range of Plant Species Initially and after Inhibition following Feeding 1 mM DCDP*

Rates were measured after equilibration (30–60 min) in air with irradiance of 1000 μE $m^{-2}s^{-1}$, then 1 mM DCDP was supplied to the cut leaf bases. Inhibition was calculated from rates measured again after 1 h compared to initial rates. The temperature in the chamber was 27 ± 2°C, the [CO_2] varied in the range 292 to 364 μL/L, and the relative humidity 24 to 70% during the experiments.

Species	Initial Photosynthesis Rate	Inhibition after DCDP (1 mM, 1 h)
	μmol $m^{-2}s^{-1}$	%
C_3 species		
Brassica napus	18.1	12
Pisum sativum	12.3	23
Hordeum vulgare	15.1	30
Triticum aestivum	19.3	46
C_4 species and subgroup		
NADP-malic enzyme-type:		
Zea mays	20.9	89
Sorghum bicolor	26.9	95
Echinochloa crusgalli	17.9	97
Phospho*enol*pyruvate carboxykinase-type:		
Panicum maximum	19.2	79
Urochloa panicoides	19.4	95
NAD-malic enzyme-type:		
Panicum miliaceum	17.8	91
Amaranthus edulis	30.6	98

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 C_4 species were selected to represent each of the three subgroups of C_4 species, defined by decarboxylation mechanism, in addition to C_3 species. Photosynthesis of all C_4 leaves was substantially inhibited by DCDP; after 1 h of feeding 1 mM DCDP, inhibition ranged from 79 to 98%. Photosynthesis in C_3 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 C_4 and C_3 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 C_3 or C_4 mechanism, with C_4 species being inhibited to a much greater extent. The results suggest that a major effect of DCDP is on the C_4 pathway and at a point which is common to all C_4 subgroups.

Since transpiration was decreased to a much lesser extent than CO_2 fixation, being only inhibited by about 40% in C_4 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 C_4 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 C_4 species was not due to some nonspecific toxicity or irreversible damage to the photosynthetic apparatus.

No recovery of photosynthesis in C_3 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 C_3 and C_4 leaves. Since PEP carboxylase is involved in stomatal opening (19) there was a possibility that DCDP inhibition in C_3 species may be due to a decreased intercellular pCO_2 caused by stomatal limitation to CO_2 diffusion. To test this, DCDP inhibition of photosynthesis was measured in C_3 leaves exposed to air containing a saturating concentration of CO_2 (0.1%) which should overcome stomatal limitations. These experiments (Table III) involved measuring control photosynthesis rates at high and normal pCO_2, then measuring rates again at each pCO_2 following a period of DCDP treatment at normal pCO_2. The results indicate that exposure to high pCO_2 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 C_3 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% CO_2 caused only about 10% inhibition of photosynthesis compared to controls (not shown).

A possible effect of DCDP on reactions of the C_3 photosynthetic carbon reduction cycle in C_4 species was also tested using isolated bundle sheath cells. Bicarbonate and light dependent photosynthetic oxygen evolution by bundle sheath

Table III. *Inhibition of Net Photosynthesis in Barley and Wheat Leaf Sections by DCDP at Normal and High pCO_2 Levels*

Photosynthesis rate was measured after equilibration of a leaf section in air containing a high pCO_2 (about 980 μbar), then after transfer to normal air (low CO_2; about 325 μbar). DCDP (1 mM) was subsequently supplied to the cut leaf base until a steady, inhibited rate was attained, then air containing high pCO_2 was supplied again and the steady rate after equilibration measured.

Treatment	Barley		Wheat	
	Photosynthesis rate	Percent of control	Photosynthesis rate	Percent of control
	μmol $m^{-2}s^{-1}$		μmol $m^{-2}s^{-1}$	
Control				
High CO_2	23.5	100	20.7	100
Low CO_2	15.8	100	14.5	100
Treated (1 mM DCDP)				
Low CO_2	10.5	66	8.5	59
High CO_2	18.7	79	16.3	79

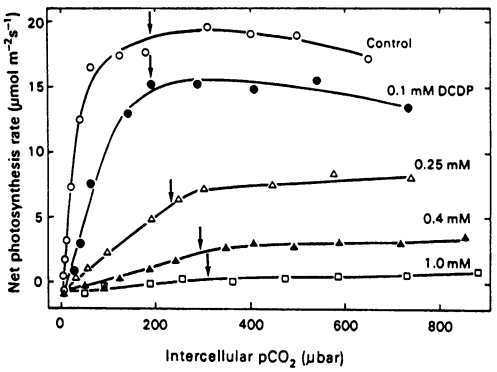

Figure 3. Response of photosynthesis rate of *P. miliaceum* leaf sections to intercellular partial pressure of CO_2 following treatment with various concentrations of DCDP. Leaf sections, with their cut bases in water, were illuminated (approximately 1000 μE $m^{-2}s^{-1}$) and net photosynthesis measured with a clamp-on leaf chamber. For each section, after a period of equilibration at [CO_2] 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 CO_2 in the supplied air was changed to 1012 μL/L and then decreased in steps, and steady-state photosynthesis rates measured. Arrows indicate intercellular pCO_2 corresponding to atmospheric [CO_2] (340 μL/L). Uninhibited rates were similar for all leaf sections. The temperature in the chamber was 27.2 ± 1°C.

strands of both *P. miliaceum* and *Urochloa panicoides* was unaffected by concentrations of DCDP up to 10 mM either included in the assay medium, or following preincubation of the strands (results not shown).

Effects of DCDP on Photosynthetic Response to CO_2 Concentration and Incident Light Intensity in *P. miliaceum*.

The response of photosynthesis of *P. miliaceum* leaf sections to the intercellular partial pressure of CO_2 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 CO_2 concentration (340 μL/L) by the amount shown (indicated by the rates at atmospheric CO_2 concentration shown by the arrows in Fig. 3). At this point the CO_2 concentration in the supplied air was increased to approximately 1012 μL/L and photosynthesis measurements at the various CO_2 concentrations begun. The high CO_2 concentrations in the air stream caused a rapid closing of stomata (stomatal conductance decreased from 293 to 118 mmol $m^{-2}s^{-1}$ 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 pCO_2) and reached a maximum at external CO_2 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 pCO_2 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 pCO_2. For example, after treatment with 1 mM DCDP, net photosynthesis was zero at a pCO_2 corresponding to atmospheric CO_2 concentration but was increased to about 1 μmol $m^{-2}s^{-1}$ (about 5% of maximum) at a pCO_2 of about 900 μbar, some fivefold higher than the pCO_2 which occurs in uninhibited leaves in normal air. It was not possible to measure photosynthesis rates at still higher pCO_2 due to equipment limitations. As a result of DCDP treatment, the apparent CO_2 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 CO_2-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 pCO_2; this effect was not further investigated.

The effect of incident light intensity on photosynthesis at near atmospheric CO_2 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 absorptance figure of 0.7, determined for the same leaf after the experiment, a quantum yield of 0.04 μmol CO_2 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

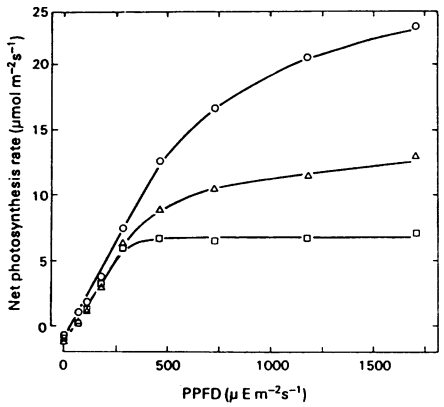

Figure 4. Effect of incident light intensity on photosynthesis rate in a *P. miliaceum* leaf initially and following feeding with DCDP. After a period of equilibration at approximately 1200 μE $m^{-2}s^{-1}$, photosynthesis rates of an excised leaf, with its cut base in water or DCDP solution, were measured 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 (○), 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°C (○), and 27.5 to 23.5°C (△,□) as light intensity decreased; the [CO_2] varied between 325 and 360 μL/L.

quantum yield is decreased (13). The lack of such an effect here suggests that no photooxidative damage has occurred.

DISCUSSION

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 C_4 and C_3 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 C_4 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 C_4 and C_3 species. While C_4 species were rapidly and sometimes completely inhibited by the compound, C_3 species were only partially inhibited. It is most likely that inhibition of photosynthesis in C_4 species occurs as a result of inhibition of PEP carboxylase. By decreasing the primary carboxylation reaction DCDP presumably limits the operation of the C_4 pathway and effectively prevents the supply of CO_2 to the bundle sheath cells. Since the PEP carboxylase reaction is common to the three C_4 subgroups, all C_4 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 C_4 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 C_3 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 C_3 PEP carboxylase (11). Although many possible functions have been suggested for PEP carboxylase in C_3 leaves a role in anapleurotic 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 CO_2 diffusion into the leaf. Consistent with this view, in the present work a partial recovery of photosynthesis was achieved by increasing the CO_2 concentration in the supplied air. In C_4 plants this explanation does not seem likely since, for *P. miliaceum*, assessment of stomatal limitation to photosynthesis based on the CO_2 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 pCO_2, at atmospheric CO_2 concentration, in a C_4 species was not decreased.

The results of Figure 3 provide a useful demonstration that CE in C_4 plants is dependent on PEP carboxylase activity, in contrast to C_3 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 K_m for HCO_3^- of PEP carboxylase or on the apparent V_{max} at saturating HCO_3^- concentration. While detailed studies on the effects of DCDP on PEP carboxylase kinetics with respect to HCO_3^- have yet to be conducted, the virtual lack of recovery of photosynthesis at high pCO_2 in the presence of DCDP argues the latter case.

The photosynthesis rate in C_4 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 C_4 pathway to supply CO_2 to the bundle sheath cells and generate a high intracellular CO_2 concentration. This capacity is given by the maximum rate which the C_4 cycle can achieve less the rate of inorganic carbon leakage from bundle sheath cells. Presumably, it is this C_4 pathway capacity that is limiting photosynthesis rates in the presence of DCDP. It seems most likely that the maximum rate at saturating CO_2 concentration is depressed because of the lowered rate of supply of CO_2 to the bundle

sheath cells and the subsequent effect of the lowering of CO_2 concentration in bundle sheath cells on Rubisco activity. The importance of the concentration of CO_2 in bundle sheath cells has been shown by Furbank and Hatch (6) who found a good correlation between the inorganic carbon pool size in C_4 leaves and the photosynthesis rate under various conditions of illumination and CO_2 concentration. Furthermore, a lowered CO_2 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 CO_2 concentration in the bundle sheath cells to prevent Rubisco oxygenase activity and the associated photorespiratory CO_2 loss.

DCDP reduced net photosynthesis in C_4 plants to zero at atmospheric CO_2 concentration (Fig. 3). Any remaining photosynthetic carbon fixation was apparently offset by respiratory carbon loss. This zero net fixation at atmospheric CO_2 concentration was increased to about 5% of the maximum uninhibited photosynthesis rate by increasing the intercellular pCO_2 approximately fivefold. It is possible that the increase may be due to direct fixation of atmospheric CO_2 by Rubisco in bundle sheath cells rather than via the normal C_4 pathway. If this is the maximal rate of photosynthesis that can be attained by direct fixation at a fivefold elevated pCO_2 in the absence of the C_4 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 C_4 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 C_4 pathway, by gaining a permeability barrier to CO_2 between mesophyll and bundle sheath cells, C_4 species have virtually lost the capability to fix atmospheric CO_2 via the usual C_3 mechanism.

The results presented here support the concept that C_4-selective herbicides may be developed based on inhibitors of C_4 pathway reactions. The PEP carboxylase inhibitor used here, when fed to leaves, decreased photosynthesis virtually to zero in C_4 species, but had lesser effects on C_3 leaves, at least in the short term. Irreversible inhibitors of PEP carboxylase, or other C_4 pathway enzymes, that could penetrate leaves would be of interest to test as potential C_4-selective herbicides.

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