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Reduced Apparent Photorespiration by the C_3-C_4 Intermediate Species, *Moricandia arvensis* and *Panicum milioides*[1]

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ABSTRACT

The CO_2/O_2 specificity factor of sucrose gradient purified ribulose 1,5-bisphosphate carboxylase/oxygenase from the C_3-C_4 intermediate plants *Moricandia arvensis* (79 ± 1) and *Panicum milioides* (89 ± 2) was similar to the respective values of the enzyme from the closely related C_3 species, *Moricandia foetida* (80 ± 5) and *Panicum laxum* (86 ± 2). Thus, the kinetic properties of this bifunctional enzyme do not explain the reduced rates of photorespiration exhibited by either of these intermediate species.

Dark/light ratios for aminoacetonitrile-sensitive $^{14}CO_2$ evolution during decarboxylation of exogenous [1-^{14}C]glycine by leaf discs had values of 9.0 with *M. arvensis* and 11.8 with *P. milioides*. Equivalent ratios with *M. foetida* and *P. laxum* were 2.5 and 3.2, respectively. Similar results were obtained using [1-^{14}C]glycolate as the exogenous photorespiratory substrate, with dark/light $^{14}CO_2$ evolution ratios for the C_3-C_4 and C_3 leaf discs averaging 6.6 and 2.0, respectively. Stimulating photosynthetic CO_2 fixation by progressively increasing photon flux density from 0 to 1900 micromoles per square meter per second caused a concomitant reduction in $^{14}CO_2$ evolution from leaf discs of *M. arvensis* and *P. milioides* supplied with [1-^{14}C]glycine. Conversely, inhibition of photosynthesis by DCMU or the Calvin cycle inhibitor DL-glyceraldehyde increased $^{14}CO_2$ evolution in the light to rates comparable to those in the dark. The data suggest that *P. milioides* and *M. arvensis* are capable of a more efficient internal recycling of photorespiratory CO_2 via ribulose bisphosphate carboxylase/oxygenase than closely related C_3 plants, and that this may partially account for the reduced rates of apparent photorespiration by these intermediate species.

Moricandia arvensis and *Panicum milioides* represent two naturally occurring terrestrial higher plants with reduced photorespiration. These species are not taxonomically related, yet show striking similarities in leaf anatomy, bundle-sheath ultrastructure, and photorespiratory CO_2 exchange characteristics which are intermediate between those of C_3 and C_4 plants (1, 3, 9, 10, 18, 27). Recent evidence obtained from (a) pulse-chase studies on incorporation of $^{14}CO_2$ into photosynthetic and photorespiratory metabolites (8, 11, 27), (b) the low activities *in vitro* of key enzymes associated with C_4 photosynthesis (8, 9, 11), and (c) immunolocalization of RuBisCO[3] and PEP carboxylase *in situ* in leaves of *P. milioides* (21) and *M. arvensis* (M. E. Salvucci and G. Bowes, unpublished) indicates that a C_4-like CO_2 concentrating mechanism is not responsible for reducing photorespiration in either of these species. In addition, based on diurnal fluctuations of malate and PEP pools, there is no evidence for the operation of CAM in leaves of *M. arvensis* (11, 27) or *P. milioides* (8). Similarly, experiments in which leaves of *M. arvensis* were treated with ethoxyzolamide (22) suggest the absence of a carbonic anhydrase-mediated active uptake system for HCO_3^- characteristic of certain aquatic photosynthetic organisms (19). Thus, no recent studies support the existence of a known biochemical CO_2 concentrating mechanism which could reduce photorespiration in either *M. arvensis* or *P. milioides* by elevating the intracellular ratio of pCO_2:pO_2 at the active site of RuBisCO.

To better understand the reduced photorespiration by these plants, we critically examined whether RuBisCO purified from either of these two intermediate species showed intrinsic differences in substrate specificity for CO_2 and O_2. Related experiments addressed the possibility that reduced apparent photorespiration by *M. arvensis* or *P. milioides* is due to slower metabolism of photorespiratory glycine (11) or a more efficient photosynthetic refixation of photorespiratory CO_2 via RuBisCO (5, 8, 27).

MATERIALS AND METHODS

Reagents. [1-^{14}C]Glycine and [1-^{14}C]glycolate were purchased from ICN and used without further purification. AAN and DL-glyceraldehyde were from Sigma.

Plant Material. *Moricandia arvensis* (L.) DC. (C_3-C_4), *Moricandia foetida* Bourg (C_3), *Panicum milioides* Nees ex. Trin. (C_3-C_4), P.I. No. 285220, *Panicum laxum* (C_3), *Panicum miliaceum* (C_4), *Glycine max* (L.) Merr. (C_3), and *Nicotiana tabacum* (C_3) were grown as described previously (9–11). Seeds of *P. laxum* and the two *Moricandia* species were kindly provided by Drs. R. H. Brown (University of Georgia) and P. Apel (Gatersleben, G.D.R.), respectively.

Purification of RuBisCO. The enzyme from market spinach (*Spinacea oleracea* L.) leaves was prepared as described by Jordan and Ogren (13). RuBisCO from each of the other species examined was prepared from young, fully expanded leaves (10 g) from 6- to 10-week-old plants. At 0 to 4°C, small leaf strips from *M. arvensis* and *M. foetida* were ground with 5 g of acid-washed sand in a pestle and mortar containing 50 ml of 100 mM Tris-

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[3] Abbreviations: RuBisCO, ribulose 1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate; PEP phosphoenolpyruvate; ethoxyzolamide, 6-ethoxy-2-benzothialesulfonamide; PVPP, polyvinylpolypyrrolidone; AAN, aminoacetonitrile; PCO cycle, photorespiratory carbon oxidation cycle; Γ, CO_2 compensation concentration.

HCl (pH 8.0 at 4°C), 2 mM Na_2EDTA, 5 mM DTT and 5% (w/v) insoluble PVPP (buffer A). Two- to 3-mm long leaf segments of the *Panicum* species were homogenized for 2 min in a Waring Blendor containing 75 ml of buffer A plus 5 mM isoascorbate and 2 mM thioglycolate (buffer B). The crude homogenate from each source was filtered through a single layer of 53-μm mesh nylon gauze. To ensure breakage of the bundle-sheath cells, residues from leaves of the *Panicum* species were each ground further in 25 ml of buffer B with a pestle and mortar, filtered as above, and the filtrates combined with those from the first homogenization step. Samples of each filtrate were fractionated between 35 and 55% saturation (4°C) with ultrapure $(NH_4)_2SO_4$ and fractions, each dissolved in 1 ml of 10 mM Bicine-NaOH, pH 8.0, 0.1 mM Na_2EDTA (buffer C), were dialyzed against the same buffer for 2 h at 4°C. Protein dialyzates, each layered above a linear sucrose density gradient comprising 0.23 to 0.67 M ultrapure sucrose in 22 ml of 20 mM Tris (pH 8.0 at 4°C), 1 mM Na_2EDTA, 2 mM $MgCl_2$, and 3 mM DTT in 2.54- × 7.62-cm polycarbonate bottles, were centrifuged for 3 h at 177,700 *g* (r_{av}) in a Beckman Ti60 fixed-angle rotor, and collected as 1.0-ml fractions. Peak fractions containing the most carboxylase activity were pooled, dialyzed overnight against buffer C plus 1 mM DTT, and stored at 4°C during the period of subsequent assays. The protein concentration in each sample was estimated colorimetrically using a protein assay kit (Bio-Rad, Richmond, CA).

RuBisCO Kinetic Constants. For $K_m(CO_2)$ determinations, enzyme (0.93–3.9 mg/ml) from each source was activated for 30 min at 25°C in 1 ml of 50 mM Bicine-NaOH (pH 8.3), 10 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ (2.1 Ci/mol), and 2730 units of erythrocyte carbonic anhydrase (Sigma). Assay solutions, in 8-ml serum-stoppered vials, were flushed for 10 min with N_2 before adding CO_2-free buffer and $NaH^{14}CO_3$. Assays were initiated by adding 20 μl of activated enzyme (containing 55 units of carbonic anhydrase) to reaction mixtures at 25°C in a total volume of 1 ml containing 50 mM Bicine-NaOH (pH 8.3), 10 mM $MgCl_2$, 0.5 mM RuBP, and 0.7 to 10.2 mM $NaH^{14}CO_3$ (2.1 Ci/mol), and terminated after 30 s by injecting 0.5 ml of 3 N HCOOH in CH_3OH. Samples were dried at 80°C and acid-stable ^{14}C dpm determined by liquid scintillation spectroscopy. Values of $K_m(CO_2)$ were estimated from a *v/s versus v* plot of the data. Lines were fitted to data by least squares analysis.

Substrate specificity factors (V_cK_o/V_oK_c), where V_c represents the V_{max} carboxylase, K_c is the $K_m(CO_2)$, V_o represents the V_{max} oxygenase, and K_o is the $K_m(O_2)$, were determined by a simultaneous radiometric assay of carboxylase and oxygenase activities essentially as described by Jordan and Ogren (12). Exceptions were that enzyme (20–78 μg) from each species was preincubated for 20 min at 25°C in reaction mixtures containing 50 mM Bicine-NaOH (pH 8.4), 10 mM $MgCl_2$, 2.5 mM $NaH^{14}CO_3$ (1.5 Ci/mol), 1.24 mM (100%) O_2, and 83 units of carbonic anhydrase. Quadruplicate assays were initiated by the addition of 0.01 mM [1-^{3}H]RuBP (9.8 Ci/mol) and terminated after 20 min by addition of 0.1 ml 0.5 N H_2SO_4/50 mM $ZnSO_4$.

Glycine and Glycolate Decarboxylation. Fully expanded leaves excised from 6- to 10-week-old plants were placed in darkness for 30 min with their bases immersed in water. An excess of discs (0.7 cm in diameter) were punched from several leaves with a sharp cork borer, and floated on distilled H_2O. Randomly selected discs were blotted dry and floated adaxial surface upwards in 25-ml Erlenmeyer flasks (6 discs/flask; 0.04–0.11 mg Chl, total) containing 0.45 to 0.95 ml of 0.3 M sorbitol, 0.3 M Mes-KOH (pH 5.5), 1 mM KH_2PO_4, and 1 mM $MgCl_2$ (buffer D). Inhibitors in buffer D, readjusted to pH 5.5, were added to give the indicated final concentrations in a total volume of 0.95 ml, and the discs were incubated in darkness at 25°C. After 1 h, the flasks were sealed by serum stoppers, to which were attached CO_2 traps comprising a filter paper wick wetted with 0.15 ml 1 M ethanolamine in a modified microcentrifuge tube. The flasks were flushed for 35 min via hypodermic needles at 50 ml/min with humidified CO_2-free air, and illuminated by sodium discharge lamps, giving a photon flux density of 1900 μmol/m^2·s PAR at the base of the flasks. Lower flux densities were provided by 150-w Westinghouse low-temperature spotlamps supplied with variable voltage by means of a rheostat. For dark assays, flasks were wrapped with aluminum foil. Decarboxylation assays, initiated by injecting 0.05 ml of 1.0 M [1-^{14}C]glycolate or [1-^{14}C]glycine (0.01 Ci/mol), were terminated after 1 h at 25°C by adding 0.5 ml 3 N HCl, completely releasing $^{14}CO_2$ from the discs and medium. After a further 30 min, the complete wick assemblies were placed in counting vials and radioactivity determined as above. Results were corrected for nonenzymic decarboxylation of substrates and a ^{14}C counting efficiency of ~60%. Adjusting the bathing medium to pH 5.5 to 6.0 enhanced the uptake of [^{14}C]glycine into the leaf discs. Preliminary experiments indicated that decarboxylation reactions were linear for at least 1 h after a slight initial lag (about 3 min). Chl was determined by the method of Wintermans and DeMots (28).

Measurement of $^{14}CO_2$ Fixation. Leaf discs in 50-ml Erlenmeyer flasks were pretreated, where indicated, with inhibitors in a total volume of 1 ml as described above. After 35 min of preillumination (1515–1900 μmol photon/m^2·s) and flushing with CO_2-free air, assay of $^{14}CO_2$ fixation was performed as described previously (7), with the exception that during the 3-min assay, $^{14}CO_2$-air was circulated at 200 ml/min via hypodermic needles passed through the serum stopper and connected by tubing to a peristaltic pump.

RESULTS AND DISCUSSION

Kinetic Properties of Partially Purified RuBisCO. The $K_m(CO_2)$ values of RuBisCO purified from *M. arvensis* and *P. milioides* were similar to the corresponding values measured for enzyme from the closely related C_3 species, *M. foetida* and *P. laxum* (Table I). These results are in accord with other comparative $K_m(CO_2)$ values published for crude RuBisCO from *M. arvensis*, *M. foetida* (2), and *P. milioides* (15, 29), and are within the range typical of the enzyme from C_3 species (29). The equivalent constant measured with RuBisCO from C_4 species has an appreciably higher value (14, 29), as indicated for *P. miliaceum* (Table I).

The CO_2/O_2 specificity factor (V_cK_o/V_oK_c) reflects the relative capacity for carboxylation and oxygenation of RuBP by RuBisCO (13, 14). For a model C_3 plant (*i.e.* devoid of a CO_2 concentrating mechanism and having insignificant rates of dark respiration in the light), the latter constant is inversely related to the magnitude of Γ. The specificity factor values of RuBisCO from *M. arvensis* and *M. foetida* were indistinguishable from the value routinely determined for the spinach enzyme, which was included as an internal C_3 control on each occasion (Table I). The average Γ values measured at 21% O_2 and 25°C were 46

Table I. *Kinetic Properties of Sucrose Gradient-Purified RuBisCO from M. arvensis, P. milioides, and Representative C_3 and C_4 Species*

Species	K_m (CO_2)[a]	CO_2/O_2 Specificity[b]
	μM	*ratio*
S. oleracea (C_3)	14.6	80 ± 2[c]
M. foetida (C_3)	16.7	80 ± 5
M. arvensis (C_3-C_4)	17.6	79 ± 1
P. milioides (C_3-C_4)	12.9	89 ± 2
P. laxum (C_3)	15.1	86 ± 2
P. miliaceum (C_4)	28.2	71 ± 1

[a] Means of duplicate assays. pK_a (HCO_3^-) = 6.23. [b] V_cK_o/V_oK_c (see Refs. 12–14 for discussion). [c] Mean ± SE.

Table II. *Decarboxylation of [1-^{14}C]Glycolate and [1-^{14}C]Glycine by Leaf Discs from M. arvensis, P. milioides, and Representative C_3 and C_4 Species*

Decarboxylation assays were performed with 50 mM [^{14}C]glycolate or [^{14}C]glycine for 1 h at pH 5.5 and 25°C, ± 1900 μmol photon/m^2·s.

Species	[1-^{14}C]Glycolate			[1-^{14}C]Glycine		
	Dark	Light	Dark/Light	Dark	Light	Dark/Light
	μmol $^{14}CO_2$ evolved·mg^{-1} Chl·h^{-1}		ratio	μmol $^{14}CO_2$ evolved·mg^{-1} Chl·h^{-1}		ratio
N. tabacum (C_3)	3.8 ± 0.21[a]	1.54 ± 0.05	2.47	3.7 ± 0.26	2.10 ± 0.06	1.76
G. max (C_3)	2.7 ± 0.19	2.26 ± 0.04	1.19	1.9 ± 0.13	0.74 ± 0.01	2.57
P. laxum (C_3)	2.4 ± 0.13	0.96 ± 0.07	2.50	3.0 ± 0.25	0.94 ± 0.06	3.19
M. foetida (C_3)				5.9 ± 0.39	2.40 ± 0.19	2.46
M. arvensis (C_3-C_4)	4.5 ± 0.18	0.82 ± 0.03	5.49	3.8 ± 0.14	0.42 ± 0.01	9.05
P. milioides (C_3-C_4)	4.2 ± 0.33	0.55 ± 0.38	7.64	4.0 ± 0.11	0.34 ± 0.06	11.8
P. miliaceum (C_4)	3.0 ± 0.14	0.14 ± 0.01	21.4	3.8 ± 0.08	~0	∞

[a] Mean ± SE of triplicate assays.

and 16 μl CO_2/L for the C_3 and C_3-C_4 intermediate *Moricandia* species, respectively (10, 11). The specificity factors of the *P. milioides* and *P. laxum* enzymes were both about 10% higher than that of spinach RuBisCO and, on this basis, a slightly lower value for Γ might be expected for both *Panicum* species in comparison to the other C_3 species examined. In the case of *P. laxum*, the measured value for Γ was in the range expected for C_3 plants (57 μl/L at 21% O_2 and 27.5°C; Ref. 18). With *P. milioides* the measured values for Γ (at 21% O_2 and 25°C, Γ = 13–19 μl CO_2/L [3, 10, 15, 18]) were 67 to 77% lower than with *P. laxum*. The lack of correlation between specificity factors and published Γ values indicates that some additional process prevents RuBisCO alone from determining the photorespiratory CO_2 exchange characteristics of these C_3-C_4 intermediate plants. Also, the similarity of $K_m(CO_2)$ and specificity factor values of RuBisCO from the C_3-C_4 intermediate species and their related C_3 control species shows that the reduced rates of photorespiration exhibited by *M. arvensis* and *P. milioides* are not explained by an altered RuBisCO enzyme with increased affinity for CO_2 relative to O_2.

Decarboxylation of [1-^{14}C]Glycolate and [1-^{14}C]Glycine. Recent pulse-chase $^{14}CO_2$ fixation experiments suggest that leaves of *M. arvensis* accumulate a larger pool of photorespiratory glycine than *P. milioides*, *M. foetida*, and *G. max.* (11). This could be due to a slower turnover rate of glycine, which might contribute to the low rates of photorespiratory CO_2 release by *M. arvensis* (10). However, experiments with leaf discs showed that the potential of *M. arvensis* to metabolize exogenously supplied [1-^{14}C]glycolate or [1-^{14}C]glycine was not markedly different from the other species examined (Table II). Rates of $^{14}CO_2$ evolution from *M. arvensis* during decarboxylation of these photorespiratory substrates in the dark were equivalent to or, in some cases, exceeded those of *P. milioides* and the four C_3 species. Preliminary experiments showed that the amount of ^{14}C present in washed leaf discs at the end of the 1-h decarboxylation assay was similar (within ± 10% SE) for each species, indicating that the comparative rates of $^{14}CO_2$ evolution were not grossly affected by differences in uptake of the labeled substrates.

Corresponding decarboxylation experiments performed at a photon flux density saturating for photosynthesis showed substantially less $^{14}CO_2$ evolution from all species than experiments performed in the dark (Table II). With either [^{14}C]glycolate or [^{14}C]glycine as substrate, ratios of $^{14}CO_2$ evolution rates in the dark *versus* the light were very much greater for the C_4 plant *P. miliaceum* than for the four C_3 species. Most notably, dark/light ratios of $^{14}CO_2$ evolution from leaf discs of *M. arvensis* and *P. milioides* were intermediate in value between those of the C_3 and C_4 plants. While $^{14}CO_2$ evolution from [^{14}C]glycine in the C_3 species was reduced by an average of 60% upon illumination, that by the C_3-C_4 intermediates was reduced by about 90%. A similar trend was observed in the decarboxylation experiments with [^{14}C]glycolate. We interpret these observations as being indicative of an enhanced capacity of the C_3-C_4 intermediate species for internal refixation of $^{14}CO_2$ produced during metabolism of the exogenous photorespiratory substrates. If the same process occurs *in vivo* during the metabolism of photosynthetically derived glycolate and glycine, it could account, at least in part, for the reduced rates of photorespiratory CO_2 evolution exhibited by these plants.

Rates of CO_2 evolution into CO_2-free air in the light are 9.0 μmol CO_2/mg Chl·h for leaves of *M. arvensis* (calculated from Ref. 10) and 5.0 to 8.5 μmol CO_2/mg Chl·h for *P. milioides* (calculated from Refs. 3 and 10), both values being appreciably greater than those presented in Table II for leaf discs. However, the concentration of exogenous [^{14}C]glycine and [^{14}C]glycolate supplied in these experiments (50 mM) was below saturating for decarboxylation reactions. In the dark, the K_m for [^{14}C]glycine decarboxylation by *M. arvensis* leaf discs was 43 mM, and $^{14}CO_2$ evolved represented 34 to 49% of the ^{14}C present in the tissue of each species after the 1-h assay in the dark.

Studies with Photosynthetic and Photorespiratory Inhibitors. Evolution of $^{14}CO_2$ in the dark from leaf discs of *M. arvensis* and *P. milioides* was inhibited by AAN (Table III, K_i ~30 mM), confirming that the observed decarboxylation of exogenous [^{14}C]glycine was associated with PCO cycle activity (26). Pretreatment of leaf discs from *M. arvensis* with increasing concentrations of DCMU progressively increased the low rates of $^{14}CO_2$ evolution during [^{14}C]glycine decarboxylation in the light to values observed in the dark (Fig. 1). At saturating levels of DCMU, the dark *versus* light ratio of $^{14}CO_2$ evolution rates from both C_3-C_4 intermediate species was close to unity (Table III). This DCMU-mediated increase in $^{14}CO_2$ evolution in the light was inversely

Table III. *Effect of Inhibitors on Decarboxylation of [1-^{14}C]Glycine by Leaf Discs from M. arvensis and P. milioides*

Decarboxylation assays were performed with 50 mM [^{14}C]glycine for 1 h at pH 5.5 and 25°C, ± 1900 μmol photon/m^2·s. Discs were preincubated with saturating concentrations of each inhibitor for 1 h in darkness.

Exp. No.	Species and Inhibitor	Dark	Light	Dark/Light
		μmol $^{14}CO_2$ evolved·mg^{-1}Chl·h^{-1}		*ratio*
M. arvensis				
1.	Control	3.69 ± 0.12	0.37 ± 0.03	9.97
2.	AAN (0.2 M)	0.68 ± 0.02	0.63 ± 0.16	1.08
3.	DCMU (0.2 mM)	5.46 ± 0.11	5.52 ± 0.28	0.99
4.	DL-glyceraldehyde (0.25 M)	2.10 ± 0.17	1.50 ± 0.17	1.40
P. milioides				
1.	Control	4.23 ± 0.18	0.44 ± 0.11	9.61
2.	AAN (0.2 M)	0.99 ± 0.17	1.04 ± 0.11	0.95
3.	DCMU (0.2 mM)	4.16 ± 0.24	4.53 ± 0.24	0.92
4.	DL-glyceraldehyde (0.25 M)	1.40 ± 0.03	1.43 ± 0.15	0.98

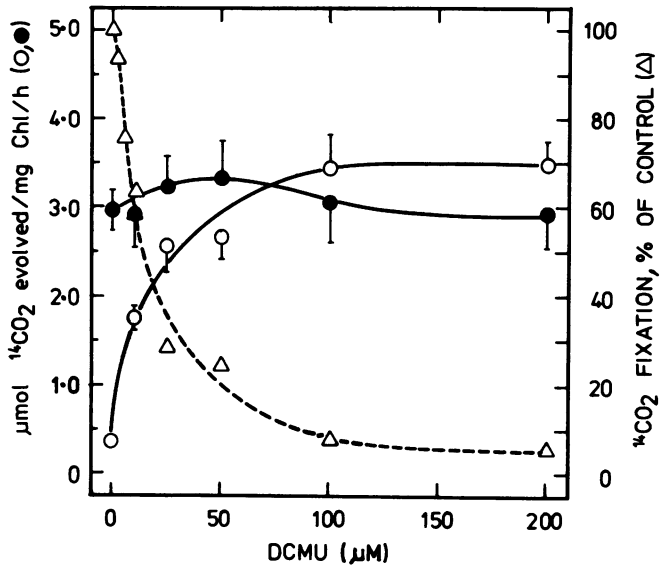

FIG. 1. Effects of DCMU on photosynthetic $^{14}CO_2$ fixation (Δ--Δ) and $^{14}CO_2$ evolution in the light (O——O; 1900 μmol photon/m^2·s) and dark (●——●) during decarboxylation of [1-^{14}C]glycine by leaf discs of *M. arvensis*. Data are the means ± SE of triplicate assays. The control rate of $^{14}CO_2$ fixation was 87.0 μmol CO_2/mg Chl·h in the presence of 340 μl $^{14}CO_2$/L and 21% O_2. Decarboxylation assays were performed with 50 mM [^{14}C]glycine for 1 h at pH 5.5 and 25°C. Discs were preincubated with DCMU for 1 h in darkness.

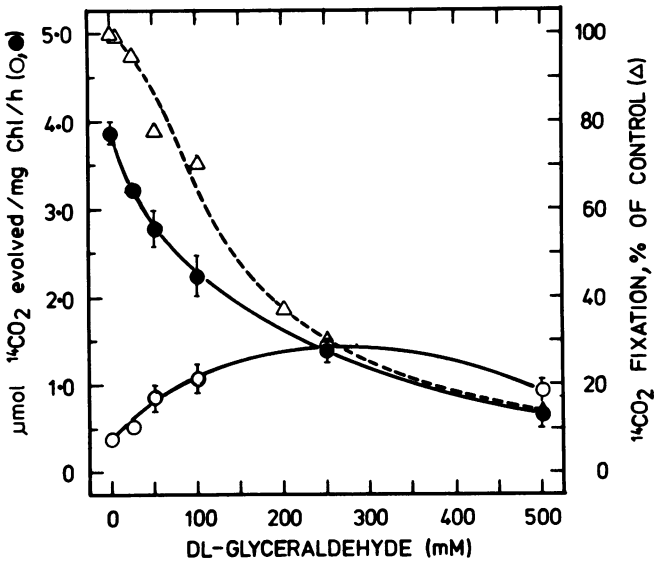

FIG. 2. Effects of DL-glyceraldehyde on photosynthetic $^{14}CO_2$ fixation (Δ--Δ) and $^{14}CO_2$ evolution in the light (O——O; 1900 μmol photon/m^2·s) and dark (●——●) during decarboxylation of [^{14}C]glycine by leaf discs of *M. arvensis*. Values are the means ± SE of triplicate assays. The control rate of $^{14}CO_2$ fixation was 76.0 μmol CO_2/mg Chl·h in the presence of 450 μl $^{14}CO_2$/L and 21% O_2. Decarboxylation assays were performed as in Figure 1 after preincubation of the leaf discs with inhibitor for 1 h in darkness.

related to the inhibition of $^{14}CO_2$ fixation by this photosynthetic electron transport inhibitor (Fig. 1). Therefore, photosynthetic refixation of $^{14}CO_2$ derived from [^{14}C]glycolate and [^{14}C]glycine fully accounts for reduced $^{14}CO_2$ evolution in the light.

In a related experiment, leaf discs from *M. arvensis* were preincubated with DL-glyceraldehyde, which inhibits photosynthesis in isolated C_3 chloroplasts by its action on the Calvin cycle enzymes transketolase and ribulose 5-P kinase (25). Treatment with this inhibitor also increased $^{14}CO_2$ evolution in the light during decarboxylation of exogenous [^{14}C]glycine (Fig. 2; Table III). As with DCMU (Fig. 1), this effect coincided with an inhibition of $^{14}CO_2$ fixation (Fig. 2), suggesting that reduced $^{14}CO_2$ evolution by the C_3-C_4 intermediate species in the light is dependent on the operation of the Calvin cycle, or more specifically, on RuBP carboxylase activity. Interpretation of the data in Figure 2 is, however, complicated by the finding that the relatively high concentrations of DL-glyceraldehyde required to inhibit photosynthesis also inhibited glycine decarboxylation *per se*, as evident from its effect on $^{14}CO_2$ evolution from leaf discs in the dark (Fig. 2; Table III). This was partially due to a reduced uptake of [^{14}C]glycine substrate into the leaf discs in the presence of the inhibitor. Similar difficulties were encountered when DL-glyceraldehyde was substituted with glycolaldehyde, which also inhibits C_3 photosynthesis by its action on Calvin cycle enzymes (24). However, the data may be conveniently analyzed by plotting the dark/light ratio for $^{14}CO_2$ evolution *versus* DL-glyceraldehyde concentration. When this is done, a normal hyperbola results (not shown).

Effect of Light Intensity. The decrease in the rate of $^{14}CO_2$ evolution from [^{14}C]glycine by leaf discs of *M. arvensis* in the light was inversely related to the rate of $^{14}CO_2$ fixation. Figure 3A shows the effects of increasing incident photon flux density from 0 to 1900 μmol/m^2·s on $^{14}CO_2$ evolution during metabolism of [^{14}C]glycine and the corresponding rates of $^{14}CO_2$ fixation

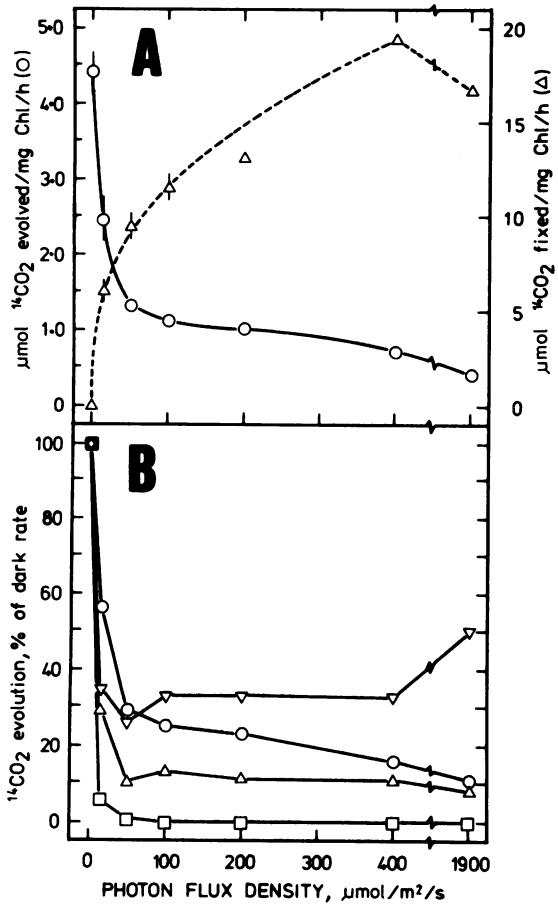

FIG. 3. A, Effect of incident light intensity on $^{14}CO_2$ evolution from leaf discs of *M. arvensis* during decarboxylation of [^{14}C]glycine (○——○), and the corresponding rates of $^{14}CO_2$ fixation in the presence of 21% O_2 and 56 μl CO_2/L (△--△). These conditions for the measurement of photosynthesis were chosen to approximate those present during determinations of Γ (see Refs. 4 and 10 and text for discussion). B, Effect of light intensity on $^{14}CO_2$ evolution from [^{14}C]glycine expressed as a percentage of the rate in the dark. (▽——▽), *N. tabacum* (C_3); (○——○), *M. arvensis* (C_3-C_4); (△——△), *P. milioides* (C_3-C_4); (□——□), *P. miliaceum* (C_4). Except for light intensity, the conditions were as described in Figure 1. Data are means ± SE of triplicate assays.

in the presence of low pCO_2 (56 μl CO_2/L). Maximal reduction of $^{14}CO_2$ evolution did not occur until light intensities were saturating for photosynthesis. To facilitate direct comparison of this light intensity effect among the C_3, C_3-C_4 intermediate, and C_4 species examined, $^{14}CO_2$ evolution rates from [^{14}C]glycine in the light were expressed as a percentage of rates in the dark (Fig. 3B). With *P. miliaceum*, a photon flux density as low as 50 to 100 μmol/m²·s was sufficient to completely prevent $^{14}CO_2$ evolution from the leaf discs, presumably due to its total refixation by both PEP and RuBP carboxylase. The $^{14}CO_2$ refixation capacity of *M. arvensis* and *P. milioides* was not markedly different from that of *N. tabacum* below 50 μmol/m²·s. At higher light intensities, however, the superiority of the two C_3-C_4 intermediate species over the C_3 control became apparent. With increasing light intensity from 50 to 1900 μmol photon/m²·s, $^{14}CO_2$ evolution by *M. arvensis* leaf discs decreased from 30% to 10% of the dark rate. This response is qualitatively similar to the influence of light intensity on Γ. Holaday *et al.* (10) noted that from 50 to 700 μmol photon/m²·s, the Γ-value of attached leaves of *M. arvensis* in 21% O_2 decreased from 44 to 14 μl CO_2/L. This range in light intensity did not affect the Γ values for the C_3 plant, *G. max* (10), suggesting that photorespiratory CO_2 loss from intact leaves of *M. arvensis* is reduced as the rate of photosynthesis increases and more CO_2 is refixed internally by RuBisCO. The parallel between the observed light intensity effects on Γ values of intact leaves (10) and $^{14}CO_2$ evolution from [^{14}C]glycine by leaf discs strengthens the physiological validity of our studies *in vitro* with exogenous photorespiratory substrates. However, although increased light intensity decreases leaf Γ values of *P. milioides* in a similar manner to those of *M. arvensis* (4, 10), no corresponding decrease was observed in $^{14}CO_2$ evolution from [^{14}C]glycine by leaf discs of *P. milioides* between 50 and 1900 μmol photon/m²·s (Fig. 3B).

CONCLUSIONS

The results of this study suggest that apparent photorespiration by the two unrelated C_3-C_4 intermediate species, *M. arvensis* and *P. milioides*, is reduced, at least in part, by photosynthetic refixation of CO_2 released during decarboxylation of photorespiratory glycine. Such internal recycling of CO_2 undoubtedly occurs to some extent in C_3 species (17), but experiments with exogenously supplied photorespiratory substrates (Table II) indicate that the two C_3-C_4 intermediate species are more efficient at this process. The absence of C_4 photosynthesis in *M. arvensis* and *P. milioides*, even at 21% O_2 and subatmospheric pCO_2 (8, 11, 27), negates any significant role for PEP carboxylase in this internal recycling of photorespiratory CO_2. Indeed, the $\delta^{13}C$ values (1, 3) and carboxylation efficiencies at 21% O_2 (R. Chollet and R. Ishii, unpublished; 16) of both species are indicative of C_3 photosynthesis. Enhancement of CO_2 release from the leaf discs of the C_3-C_4 intermediate species in the light by application of the Calvin cycle inhibitor, DL-glyceraldehyde (Fig. 2; Table III) indicates that photorespiratory CO_2 is refixed primarily by RuBisCO. It has been suggested (19) that, in *P. milioides* and *M. arvensis*, RuBisCO would not be capable of reducing photorespiratory CO_2 loss in this manner unless its affinity for substrate CO_2 relative to O_2 were markedly greater than the enzyme from C_3 plants. Yet, determination of CO_2/O_2 specificity factors of RuBisCO purified from *M. arvensis* and *P. milioides* showed no significant differences from closely related C_3 species (Table I). Also, since RuBisCO is not confined to the bundle-sheath chloroplasts in either intermediate species (M.E. Salvucci and G. Bowes, unpublished; 21) as in C_4 plants, the intriguing question arises as to how these species might achieve low rates of apparent photorespiration by the CO_2 recycling mechanism suggested.

Ultrastructural studies of leaves of C_3-C_4 intermediate species, including *M. arvensis* and *P. milioides*, have led to speculation on the feasibility of enhanced photosynthetic recycling of photorespiratory CO_2 via RuBisCO (5, 8, 27). Most notably, a quantitative ultrastructural analysis of *Panicum* species in the *Laxa* group (5) showed the bundle-sheath cells of *P. milioides* to contain a significantly greater proportion of the total leaf mitochondria, peroxisomes, and chloroplasts than bundle-sheath cells of the C_3 plant, *P. laxum*. Possibly, a large proportion of CO_2 photorespired during glycine metabolism in the mitochondria of this cell-type is refixed by RuBisCO in the closely associated bundle-sheath chloroplasts (5, 9, 27) and surrounding mesophyll cells before it can exit the leaf. However, it remains to be established whether the amount of photorespiratory CO_2 produced in the bundle-sheath cells is sufficient to influence whole leaf CO_2 exchange.

In C_4 plants, the glycine oxidation system is localized exclusively in the mitochondria of the bundle-sheath cells (20). If a similar compartmentation of glycine decarboxylase were to occur in the C_3-C_4 intermediate species, it could result in an enrichment of photorespired CO_2 at the interior of the leaf, improving the chances of refixation. However, in the absence of any corresponding intercellular compartmentation of RuBisCO, it seems

unlikely that the necessary mass movement of photorespiratory intermediates would occur between the mesophyll and bundle-sheath cells.

Refixation of photorespired CO_2 in *M. arvensis* and *P. milioides* probably does not totally account for the reduced rates of photorespiration by these plants. Preliminary CO_2 exchange measurements on *P. milioides* performed by differential $^{14}CO_2/^{12}CO_2$ uptake (6) suggest that true photosynthesis by *P. milioides* is less sensitive to inhibition by atmospheric levels of O_2 than that of the C_3 plant *P. bisulcatum* (D. T. Canvin, unpublished). Thus, at ambient pCO_2, RuBisCO may be insulated from external oxygen concentrations of up to 21%, possibly by an internal pool of CO_2. Servaites *et al.* (23) studied comparative rates of glycolate accumulation during photosynthesis in $^{14}CO_2$-air by *Hordeum vulgare* (C_3), *P. milioides*, and *P. miliaceum* (C_4) treated with butyl hydroxybutynoate, an inhibitor of glycolate oxidase. Relative to the C_3 and C_4 species, leaves of *P. milioides* exhibited intermediate rates of [^{14}C]glycolate production based on the percentage distribution of total ^{14}C fixed. This observation is consistent with a reduced entry of carbon into the PCO cycle due to an elevated CO_2/O_2 ratio at the active site of RuBisCO, since this bifunctional enzyme is not altered kinetically (Table I). Further studies are needed to evaluate the contribution of CO_2 recycling, as well as the existence of other mechanisms to reduce photorespiration in these C_3-C_4 intermediate species.

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