Degree of C₄ Photosynthesis in C₄ and C₃-C₄ Flaveria Species and Their Hybrids

I. CO₂ Assimilation and Metabolism and Activities of Phosphoenolpyruvate Carboxylase and NADP-Malic Enzyme


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ABSTRACT

The degree of C₄ photosynthesis was assessed in four hybrids among C₄, C₃-like, and C₃-C₄ species in the genus Flaveria using ¹⁴C labeling, CO₂ exchange, ¹³C discrimination, and CO₃ enzyme activities. The hybrids incorporated from 57 to 88% of the ¹⁴C assimilated in a 10-s exposure into C₄ acids compared with 26% for the C₄-C₃ species Flaveria linearis, 91% for the C₃ species Flaveria trinervia, and 87% for the C₃-like Flaveria brownii. Those plants with high percentages of ¹⁴C initially fixed into C₄ acids also metabolized the C₄ acids quickly, and the percentage of ¹⁴C in 3-phosphoglyceric acid plus sugar phosphates increased for at least a 30-s exposure to ¹⁴CO₂. This indicated a high degree of coordination between the carbon accumulation and reduction phases of the C₄ and C₃ cycles. Synthesis and metabolism of C₄ acids by the species and their hybrids were highly and linearly correlated with discrimination against ¹³C. The relationship of ¹³C discrimination or ¹³C metabolism to O₂ inhibition of photosynthesis was curvilinear, changing more rapidly at C₃-like values of ¹³C metabolism and ¹³C discrimination. Incorporation of initial ¹⁴C into C₄ acids showed a biphasic increase with increased activities of phosphoenolpyruvate carboxylase and NADP-malic enzyme (steep at low activities), but turnover of C₄ acids was linearly related to NADP-malic enzyme activity. Several other traits were closely related to the in vitro activity of NADP-malic enzyme but not phosphoenolpyruvate carboxylase. The data indicate that the hybrids have variable degrees of C₄ photosynthesis but that the carbon accumulation and reduction portions of the C₄ and C₃ cycles are well coordinated.

The CO₂ assimilation pathway of C₄ species is generally well understood. The higher CO₂ assimilation capacity and lack of photorespiration in these species are the result of high CO₂ in BSC² (11). The completely developed C₄ pathway requires high activity levels of the enzymes in the pathway.

1 Supported by state and Hatch funds allocated to the University of Georgia.

2 Abbreviations: BSC, bundle sheath cells; AP, apparent photosynthesis; (Table 1) CO₂ compensation concentration; 3-PGA, 3-phosphoglyceric acid; NADP-ME, NADP-malic enzyme; PEPcase, phosphoenolpyruvate carboxylase.

MATERIALS AND METHODS

Plant Materials

Four hybrids were made as described earlier (2, 12). Two of the hybrids (Flaveria trinervia [C₃] × Flaveria brownii × F. linearis) have been described in terms of CO₂ exchange, leaf anatomy, carboxylase activities, and cytogenetics (2, 4, 12). In the following experiments, we used these two hybrids plus two hybrids made by crossing the F₁, F. brownii × F. linearis, with F. trinervia, using F. trinervia as the maternal parent. Several hybrids were made using this parental combination, and two hybrids (designated 872-522 (designated 872-522).
and 87–527) were selected for further study based on their contrasting response of AP to O2. The hybrid 87–522 was inhibited by only 11% by 210 mL L−1 of O2, whereas in 87–527 the inhibition was about 20%.

Plants were grown in the greenhouse using supplemental light during part of the day to ensure minimum irradiance at midday of 1.5 mmol quanta m−2 s−1. Temperatures in the greenhouse were maintained at 30 to 35°C during the day and 20 to 25°C at night. Plants were grown in 2- to 4-L pots (depending on plant size) in soil, peat, and perlite (1:1:1 mixture) and fertilized two to three times weekly with Hoagland solution. Plants were transferred to the laboratory just before use.

**CO2 Exchange**

Measurements of AP and O2 inhibition of AP were made as described previously (2). Attached leaves were enclosed in the leaf chambers and exposed to 335 μL L−1 of CO2 and 210 mL L−1 of O2. When steady rates of CO2 exchange were obtained, the O2 concentration was reduced to 20 mL L−1, and AP was measured again. Rates of AP measured in this way were used to calculate O2 inhibition of AP. Leaf temperature was maintained at 30°C. Dew point of gas entering the chamber was 12.7°C, and transpiration increased the dew point of exhaust air to 15 to 20°C. Irradiance on the leaves was maintained at 2.0 mmol quanta m−2 s−1 (400–700 nm). Young fully expanded leaves were used (usually the fourth leaf from the stem apex). Single leaves from three vegetatively propagated plants of each parent and hybrid were measured in each of two experiments.

Measurements of I were made at the completion of AP measurements in the second experiment. A young fully expanded leaf at the fourth node from the stem apex was placed in a 20-mL circular chamber while still attached to the stem. The chamber was flushed with air having a low CO2 concentration (approximately 50 μL L−1) via two three-way electronic valves located at opposite sides of the chamber. With the valves at the “off” position, CO2-free air bypassed the chamber and entered an IRGA. After the leaf was equilibrated for 20 min, the valves were activated, and the chamber was flushed with CO2-free air, which entered the IRGA. The residual CO2 in the chamber registered as a peak on the IRGA and connected recorder. The peak height was compared with calibrated peaks obtained by flushing the chamber without a leaf after filling with known CO2 concentrations. Measurements were made at 30°C, 210 mL L−1 of O2, and an irradiance of 2.0 mmol quanta m−2 s−1. Leaves from three plants vegetatively propagated from each parent and hybrid were tested, except for *F. trinervia* × *F. linearis*, which was not tested.

**14CO2 Assimilation**

Two experiments on separate sets of vegetatively propagated plants were conducted to determine 14C-labeling patterns and turnover of photosynthetic products. Leaves at the third or fourth node from the stem tip were detached by cutting under water and transferred to a 54-cm3 leaf chamber made of 3-cm diameter glass tubing with a rubber stopper in each end. Each stopper had two holes, one of which was fitted with glass tubing for passing air through the chamber. The other hole in the bottom stopper was fitted with a small water reservoir to hold the detached leaf base. The second hole in the top stopper had a short glass tube fitted with a rubber septum for injection of 14CO2.

Before each labeling with 14CO2, a leaf was placed in the chamber, and the system was flushed at 0.5 L min−1 with air containing 330 to 380 μL L−1 of CO2 and humidified to a dew point of 15 to 17°C. The chamber air and a reference sample that bypassed the chamber were passed through cells of an IRGA, and the CO2 differential was monitored until a steady AP was obtained. This usually required from 30 to 60 min. When a steady CO2 uptake rate was obtained, flow through the chamber was stopped, and tubing clamps were used to seal the chamber. The top stopper was removed, and the chamber was flushed for about 15 s with air containing 90 μL L−1 of CO2. The top stopper was then replaced and 14CO2 added by injection from a syringe to bring the total CO2 concentration to 400 μL L−1. Based on measured CO2 uptake and leaf size, it was calculated that CO2 concentration decreased 30 to 100 μL L−1 during the 10-s labeling period. Leaf temperature was held at 25 ± 1.0°C, and irradiance was 1070 μmol quanta m−2 s−1 (400–700 nm).

The 14CO2 used in labeling was generated by placing a NaH14CO3 solution (56 Ci/mol) in a flask that had been flushed with CO2-free air and injecting concentrated H3PO4. Small samples of 14CO2-air (4 mL) were withdrawn from the flask and injected into the 14CO2-labeling chamber. After a 10-s pulse of 14CO2, the leaf was either quickly removed from the chamber with forceps and killed immediately or subjected to 12CO2 in air at 330 to 380 μL L−1 of CO2 for various chase periods and then killed. During the 12CO2 chase, the chamber was flushed at about 10 L min−1.

Leaves were killed by plunging them into boiling 80% (v/v) ethanol in the first experiment and liquid N2 in the second experiment. In the second experiment, leaves were crushed in liquid N2, and the frozen powder was sprinkled in boiling 80% (v/v) ethanol. Leaves were extracted by boiling in the ethanol for 5 to 10 min. In the first experiment, leaves were ground in the ethanol with a mortar and pestle and washed twice with 80% (v/v) ethanol. The mixture was filtered and then concentrated to 5 to 10 mL at 40°C with a rotary evaporator. The solutions were then partitioned with petroleum ether, and the aqueous phase was evaporated to dryness in a freeze dryer and then dissolved in 1 mL of water. In experiment 2, the extract was centrifuged after boiling, and the supernatant fluid was partitioned with petroleum ether and freeze dried as in experiment 1.

14C-Labeled compounds were separated by paper chromatography using procedures described earlier (5). Aliquots containing 30,000 cpm or more were spotted on the chromatographs, which were developed first in phenol:water (72:28, v/v) and then in the second direction in 1-butanol:propionic acid:water (46:23:31, v/v). Chromatograms were exposed to no-screen x-ray film, and the spots located were cut out and counted by liquid scintillation counting. Known 14C-labeled compounds were used as standards.

Chase times in 12CO2 were 5, 15, 30, 60, and 180 s in experiment 1 and 30 and 60 s in experiment 2. Results were
very similar for the two experiments, and only the 30-, 60-, and 180-s chase times are presented. The 10-s 14CO2 pulse and the 30- and 60-s chase times are averaged for the two experiments.

13C Analysis

The 13C/12C ratios were determined at the Center for Applied Isotope Studies at the University of Georgia on leaves used for CO2 exchange measurements. The leaves were detached, dried at 70°C for 36 h, and finely ground. The replicate leaves were combined before grinding. The 13C/12C ratios are expressed as δ13C, where δ13C = ([Rsample/Rstandard] − 1) × 1000 and R is the ratio 13C/12C. The standard is PeeDee belemnite carbonate. The precision ranges from ±0.02 to 0.20‰.

Enzyme Activity

Preparation of leaf extracts and determination of activities of PEPcase and NADP-ME from young, fully expanded leaves were according to the procedure described earlier (4). Extracts of previously determined PEPcase and NADP-ME activity were included as controls to monitor efficiency of extraction and assay performance. Chl was extracted (22) from strips cut from the middle of the leaf that was subsequently used for enzyme assays; Chl a and Chl b were assayed according to the method of Inskeep and Bloom (15). Because Chl and enzymes were assayed on separate leaf material, they were normalized to g fresh weight to express activity on a per milligram Chl basis.

RESULTS

CO2 Exchange

Various aspects of CO2 exchange have been reported for these species and two of the hybrids (2, 4, 12). Inhibition of AP by 210 mL L−1 of O2 for F. trinervia × F. linearis and F. brownii × F. linearis hybrids was reported to be between that of the parents (4, 12). However, in the present experiments, F. trinervia × F. linearis (Table I) and F brownii × F. linearis exhibited O2 inhibition values similar to that of F. linearis. The two three-way hybrids of F. trinervia × (F. brownii × F. linearis) differed in O2 inhibition, with AP of 87–522 being inhibited only about 11% by atmospheric O2 levels and that of 87–527 having about 20% inhibition, similar to that of F. linearis. The 87–527 hybrid also exhibited only about 50% as high AP as 85–522.

The ranking among the plants for δ13C of leaves tended to follow O2 inhibition of AP (Table I). Values for the F1 hybrids were more positive than those for the C3-C4 parent but more negative than the C3 and C4-like parents. The F1 hybrid involving F. brownii was 2.1‰ more negative than the one with F. trinervia, although F. brownii was only slightly more negative (0.3‰) than F. trinervia. When the F. brownii × F. linearis hybrid was crossed with F. trinervia, δ13C of the resulting hybrids was between those of the parents. The two three-way hybrids differed with respect to δ13C, with 87–522 being 2.7‰ higher than 87–527. Values of Γ were C4-like for all of the plants except F. linearis, which had a Γ of 14 ± 3 μL L−1.

<table>
<thead>
<tr>
<th>Table I.</th>
<th>AP, O2 Inhibition of AP, Γ, and δ13C for F. trinervia (C3), F. brownii (C4-like), F. linearis (C3-C4), and Hybrids among the Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species/Hybrids</td>
<td>Experiment</td>
</tr>
<tr>
<td>F. trinervia</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>F. brownii</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>F. linearis</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>F. brownii × F. linearis</td>
<td>1</td>
</tr>
<tr>
<td>F. trinervia × (F. brownii × F. linearis)</td>
<td>87–522</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>87–527</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>F. trinervia × F. linearis</td>
<td>21 ± 3</td>
</tr>
</tbody>
</table>

* O2 inhibition is calculated as (AP at 20 mL L−1 of O2 − AP at 210 mL L−1 of O2)/AP at 20 mL L−1 of O2 × 100. * Data for this hybrid are from a separate set of experiments. Values for F. trinervia and F. linearis from the separate experiment are not shown but were very similar to values in experiment 1.
14CO2 Assimilation and Turnover

F. trinervia and F. brownii incorporated about 90% of the 14C assimilated into C4 acids in 10 s (Fig. 1, A and B). In contrast, F. linearis incorporated only 26% of the total 14C into C4 acids (Fig. 1C). The interspecific hybrids incorporated varying percentages of 14C in C4 acids, ranging from 57% in F. brownii × F. linearis to 88% in hybrid 87–522 (Fig. 1, D–G). Hybridizing F. trinervia with F. linearis resulted in more incorporation of 14C in C4 acids (70%) than when F. brownii was crossed with F. linearis (57%) (Fig. 1, E and F).

The decrease in percentage of 14C in C4 acids during a 60-s chase with 12CO2 tended to rank among the genotypes in the same way as 14C incorporation into C4 acids. The percentage of 14C in C4 acids decreased about 55 units in F. trinervia, F. brownii, and the 87–522 hybrid (Fig. 1, A, B, and D). The smallest turnover was only 5.4% in F. linearis (Fig. 1C). Although hybrids 87–522 and 87–527 assimilated almost equal percentages of 14C in C4 acids in 10 s, turnover of 14C-labeled C4 acids was only one-half as fast in 87–527 as in 87–522 (27 versus 54 percentage units) (Fig. 1, D and G).

Figure 1. Percentage of 80% (v/v) ethanol-soluble 14C in various metabolites from three Flaveria species and their hybrids following exposure to 14CO2 for 10 s and subsequent exposure to 12CO2 for 30, 60, and 180 s. Leaves were exposed to 330 to 380 µL L−1 of CO2, 1070 µmol quanta m−2 s−1, and 25°C. Points at 0, 30, and 60 s are means of two experiments, whereas those at 180 s are from a single experiment. Sug., Sugar.
Turnover of \(^{14}\text{C}\)-labeled \(\text{C}_4\) acids was slower in \(F.\ brownii \times F.\ linearis\) (18 units) (Fig. 1E) than in the other three hybrids.

The rapid decrease in \(^{14}\text{C}\) in \(\text{C}_4\) acids in \(\text{C}_4\) species is accompanied by an increase in \(^{14}\text{C}\) in 3-PGA plus sugar phosphates as carbon is transferred from initial products to the reductive pentose pathway products in BSC (11). An increase in \(^{14}\text{C}\)-labeled 3-PGA plus sugar phosphates in 30 s of \(^{13}\text{CO}_2\) exposure occurred in those plants that showed large decreases (25\% or more in 30 s) in \(^{14}\text{C}\) in \(\text{C}_4\) acids (Fig. 1, A, B, and D). Among the seven species and hybrids, the decrease in percentage of \(^{13}\text{C}\) in \(\text{C}_4\) acids in 30 s was highly correlated with the change in \(^{14}\text{C}\) in 3-PGA plus sugar phosphates \((r = 0.93,\) data not shown). The correlation was somewhat lower \((r = 0.81)\) between \(^{14}\text{C}\) initially incorporated in \(\text{C}_4\) acids and the change in \(^{14}\text{C}\) in 3-PGA plus sugar phosphates in 30 s (data not shown).

**Correlations among Traits**

Except for \(\Gamma\), which did not vary much among the plants, and PEPcase activity, which varied widely (see below and Fig. 4C), there were clear relationships among the photosynthetic traits measured. When \(^{13}\text{C}\) values for the species and hybrids were plotted against \(\text{C}_4\) acid pulse labeling and turnover and against the change in \(^{14}\text{C}\) in 3-PGA plus sugar phosphates, linear relationships were obtained with respective correlation coefficients of 0.96, 0.97, and 0.88 (Fig. 2).

The \(\text{O}_2\) inhibition of AP was related in a curvilinear way to both the initial percentage of \(^{14}\text{C}\) incorporated into \(\text{C}_4\) acids and the \(^{13}\text{C}\) values of leaves of \(\text{Flaveria}\) species and hybrids (Fig. 3). The \(\text{O}_2\) inhibition of AP decreased as both \(^{13}\text{C}\) and the percentage of \(^{14}\text{C}\) in \(\text{C}_4\) acids increased. The change in \(\text{O}_2\) inhibition with changes in these parameters was less at low (more \(\text{C}_4\)-like) values and greater at more \(\text{C}_4\)-like values. All of the data fit the curve well except for the hybrid 87–527, which had higher \(\text{O}_2\) inhibition of AP than would be predicted from the relationships among the parents and other hybrids.

Activity of NADP-ME was more closely related to other photosynthetic traits than was that of PEPcase. Except for initial \(^{14}\text{C}\) incorporated into \(\text{C}_4\) acids (Fig. 4C), PEPcase activity was not closely associated with any other of the parameters measured. Activity of PEPcase was also not related to NADP-ME activity \((r = 0.49)\). On the other hand, NADP-ME showed a close association with \(^{13}\text{C}\), initial \(^{14}\text{C}\) incorporated into \(\text{C}_4\) acids, and loss of \(^{13}\text{C}\) from \(\text{C}_4\) acids (Fig. 4). In addition, NADP-ME activity was linearly related to \(\text{O}_2\) inhibition of AP \((r = -0.93,\) data not shown).

**DISCUSSION**

The degree of inheritance of \(\text{C}_4\) photosynthesis by interspecific \(\text{Flaveria}\) hybrids reported earlier (4, 12) has been further investigated using \(^{14}\text{CO}_2\) labeling of photosynthetic metabolites. The intermediate values of \(\text{O}_2\) inhibition of AP and \(^{13}\text{C}\) reported earlier (2, 12) were confirmed and found to be correlated with initial labeling and turnover of \(\text{C}_4\) acids. The apparent degree of \(\text{C}_4\) photosynthesis in the hybrids varied with the completeness of the \(\text{C}_4\) syndrome in the parents. In hybrids of \(F.\ trinervia \times F.\ linearis\), \(^{13}\text{C}\) was more positive, more \(^{14}\text{C}\) was incorporated initially into \(\text{C}_4\) acids, and turnover of \(\text{C}_4\) acids was faster than in hybrids of \(F.\ brownii \times F.\ linearis\). Likewise, in hybrids of \((F.\ brownii \times F.\ linearis) \times F.\ trinervia\), the above traits became more like \(\text{C}_4\) with the exception of \(\text{O}_2\) inhibition of AP in 87–527, which was similar to that of both \(F.\ linearis\) and \(F.\ brownii \times F.\ linearis\).

The lower degree of \(\text{C}_4\) photosynthesis in the \(F.\ brownii \times F.\ linearis\) hybrid compared with \(F.\ trinervia \times F.\ linearis\) is not surprising in view of earlier studies showing incomplete development of \(\text{C}_4\) photosynthesis in \(F.\ brownii\) (7, 17). However, in our earlier gas exchange studies, \(F.\ brownii \times F.\ linearis\) had \(\text{CO}_2\) exchange traits and \(^{13}\text{C}\) values equivalent to \(F.\ trinervia \times F.\ linearis\) (1, 4, 12). Likewise, in the work reported here, \(F.\ brownii\) had values equivalent to \(F.\ trinervia\).
Thus, Flaveria floridana, initial photosynthesis depends on brownii as is 87–522. However, brownii x 0.3%o phosphates sugar for their hybrids. Figure 3 illustrates a recombination of genes controlling C4 photosynthesis. In every trait tested except PEP-case activity, 87–522 was more like C4 than 87–527. Although the difference in percentage of initial 14C incorporated into C4 acids differed only slightly (88 versus 83%), differences in 14C loss from C4 acids and its appearance in 3-PGA plus sugar phosphates were substantial. The more coordinated C4 photosynthesis in 87–522 was substantiated by more positive δ13C values compared with 87–527. In fact, based on the 14CO2 incorporation data, it appears that C4 photosynthesis has been largely restored in 87–522 by hybridizing F. brownii for 14C incorporation in C4 acids, loss of 14C from C4 acids during the 13CO2 chase, and increase of 14C in 3-PGA plus sugar phosphates (Fig. 1, A and B). In addition, δ13C was only 0.3‰ more positive in F. trinervia than F. brownii (Table I). However, despite the nearly complete C4 cycle based on the present work and CO2 exchange characteristics from other studies (4, 6, 12, 17), F. brownii is not able to pass on to its progeny the same degree of C4 acid synthesis and metabolism as is F. trinervia. It is interesting to note that in F. brownii x Flaveria floridana, a C3-C4 species with a higher degree of C4 photosynthesis than F. linearis, the F1 hybrids exhibited a higher initial 14C labeling of C4 acids than we observed for F. brownii x F. linearis (6). In fact, one of the reciprocal hybrids had a higher percentage of 14C in C4 acids (85%) than F. brownii (77%), and the other had an initial percentage of 66. Thus, the degree of C4 photosynthesis in hybrids apparently depends on the degree in both parents.

The difference between the two three-way hybrids, 87–522 and 87–527, illustrates a recombination of genes controlling C4 photosynthesis. In every trait tested except PEP-case activity, 87–522 was more like C4 than 87–527. Although the difference in percentage of initial 14C incorporated into C4 acids differed only slightly (88 versus 83%), differences in 14C loss from C4 acids and its appearance in 3-PGA plus sugar phosphates were substantial. The more coordinated C4 photosynthesis in 87–522 was substantiated by more positive δ13C values compared with 87–527. In fact, based on the 14CO2 incorporation data, it appears that C4 photosynthesis has been largely restored in 87–522 by hybridizing F. brownii.
× *F. linearis* with *F. trinervia*. However, O$_2$ inhibition of AP in 87–522 is about midway between the parents, and δ$^{13}$C is 2.0% more negative than in *F. trinervia*. In addition, an inhibitor of PEPcase activity reduced AP by only 52% in hybrid 87–522 compared with 90% in *F. trinervia* (3). Thus, a high initial percentage of $^{14}$C in C$_4$ acids and rapid metabolism are apparently not enough to ensure complete manifestation of C$_4$ photosynthesis.

Discrimination against $^{13}$C in CO$_2$ assimilation may be the best indicator of the degree of C$_4$ photosynthesis. In C$_3$ species, the main discrimination is by Rubisco, but in C$_4$ species, this enzyme is restricted to BSC, and the low CO$_2$ permeability of BSC reduces the chance for discrimination against $^{13}$C (10). A futile cycle of phosphoenolpyruvate carboxylation and decarboxylation as described by Monson et al. (18) could allow substantial levels of $^{14}$C labeling of C$_4$ acids but without an equivalent degree of integrated C$_4$ photosynthesis. Values of δ$^{13}$C, on the other hand, probably reflect the proportion of carbon that is assimilated by C$_4$ photosynthesis into dry matter. The strong linear relationship obtained between δ$^{13}$C and initial $^{14}$C incorporation in C$_4$ acids, the loss of $^{14}$C from C$_4$ acids, and the increase in 3-PGA plus sugar phosphates early in the chase period (Fig. 2) show that δ$^{13}$C is a good indicator of the level of C$_4$ cycle activity. However, it is interesting to note that δ$^{13}$C values for *F. linearis* are usually no different from those for C$_3$ species (2, 12), and Monson et al. (19) found little variation in δ$^{13}$C among C$_3$–C$_4 *Flaveria* species, which exhibited a range in other traits related to C$_4$ photosynthesis.

Plots of O$_2$ inhibition of AP against initial $^{14}$C in C$_4$ acids (Fig. 3B) suggest that the relationship is less direct than with δ$^{13}$C and that the predictive value of O$_2$ inhibition of AP for C$_4$ photosynthesis is not as good. The curvature of the relationship between O$_2$ inhibition and initial $^{14}$C in C$_4$ acids suggests that O$_2$ inhibition of AP does not change very greatly until C$_4$ photosynthesis is nearly complete. This was also reflected in the sharp decrease in O$_2$ inhibition of AP when δ$^{13}$C approached C$_4$-like values (Fig. 3A). Inhibition of AP by O$_2$ is influenced not only by the proportion of CO$_2$ assimilated by the C$_4$ cycle but also by the strict compartmentation of glycine decarboxylase in BSC and refixation of photorespired CO$_2$ as occurs in C$_3$–C$_4$ species of *Flaveria* and in other C$_3$–C$_4$ species that lack C$_4$ acid metabolism (14). In addition, O$_2$ inhibition of AP is influenced by the CO$_2$ permeability of BSC. Thus, O$_2$ sensitivity may decrease greatly when enzyme partitioning and BSC impermeability are nearly complete.

The biphasic change in initial $^{14}$C incorporation into C$_4$ acids with increased PEPcase activity (Fig. 4C) indicates that high levels of activity are not required for C$_4$-like labeling of C$_4$ acids. Activities of NADP-ME also showed a biphasic relationship with $^{14}$C labeling in C$_4$ acids, but turnover of the C$_4$ acids was linearly related to NADP-ME activity (Fig. 4B). This linear relationship might be expected because this enzyme decarboxylates malate in C$_3$ and C$_3$–C$_4$ species of *Flaveria*, but it also may mean that the degree of C$_4$ photosynthesis in this group of plants is dependent on this enzyme because of its role in concentrating CO$_2$ in BSC. This dependency is reinforced by the close relationship between NADP-ME activity and δ$^{13}$C (Fig. 4A).

The activities of PEPcase and NADP-ME reported in Figure 4 are approximately one-third of values previously reported in the literature (17). This is not due to suboptimal assay conditions, because both activities of PEPcase per milligram of protein and per gram fresh weight in the C$_4$ species, *F. trinervia*, are comparable to other published values (data not shown). Plotting the parameters in Figure 4 on the basis of micromoles per gram fresh weight per hour or micromoles per milligram of protein per hour does not change the relationships shown in Figure 4. The difference in our values and those of others most likely reflects the greater efficiency and stability of extracting Chl in dimethylformamide (22).

The data reported here show that interspecific *Flaveria* hybrids result in varying degrees of C$_4$ acid metabolism, which is strongly correlated with δ$^{13}$C values of leaves. This is in contrast to the variable C$_4$ acid metabolism among the several naturally occurring C$_3$–C$_4 *Flaveria* species, which apparently is unrelated or only weakly associated with δ$^{13}$C values (18, 19, 21). This may mean that the CO$_2$ fixation and reduction portions of the C$_4$ cycle are rather well coordinated in the four hybrids, even though they differ in levels of C$_4$ acid metabolism.

**LITERATURE CITED**

exchange, cytogenetics, and leaf anatomy of hybrids between photosynthetically distinct Flaveria species. Plant Physiol 89: 839–844