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Photosynthetic Induction in a C_4 Dicot, *Flaveria trinervia*[1]

I. INITIAL PRODUCTS OF $^{14}CO_2$ ASSIMILATION AND LEVELS OF WHOLE LEAF C_4 METABOLITES

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BRANDON D. MOORE* AND GERALD E. EDWARDS
Department of Botany (B.d.M., G.E.E.) *and Institute of Biological Chemistry* (G.E.E.), *Washington State University, Pullman, Washington 99164-4230*

ABSTRACT

Labeling patterns from $^{14}CO_2$ pulses to leaves and whole leaf metabolite contents were examined during photosynthetic induction in *Flaveria trinervia*, a C_4 dicot of the NADP-malic enzyme subgroup. During the first one to two minutes of illumination, malate was the primary initial product of $^{14}CO_2$ assimiltion (about 77% of total ^{14}C incorporated). After about 5 minutes of illumination, the proportion of initial label to aspartate increased from 16 to 66%, and then gradually declined during the following 7 to 10 minutes of illumination. Nutrition experiments showed that the increase in $^{14}CO_2$ partitioning to aspartate was delayed about 2.5 minutes in plants grown with limiting N, and was highly dampened in plants previously treated 10 to 12 days with ammonia as the sole N source. Measurements of C_4 leaf metabolites revealed several transients in metabolite pools during the first few minutes of illumination, and subsequently, more gradual adjustments in pool sizes. These include a large initial decrease in malate (about 1.6 micromoles per milligram chlorophyll) and a small initial decrease in pyruvate. There was a transient increase in alanine levels after 1 minute of illumination, which was followed by a gradual, prolonged decrease during the remainder of the induction period. Total leaf aspartate decreased initially, but temporarily doubled in amount between 5 and 10 minutes of illumination (after its surge as a primary product). These results are discussed in terms of a plausible sequence of metabolic events which lead to the formation of the intercellular metabolite gradients required in C_4 photosynthesis.

Photosynthetic induction is a period during which metabolic transitions occur upon the illumination of dark-adapted plants. During the induction process there is an initial lag in photosynthetic rates. In C_4 species, maximum activation of specific photosynthetic enzymes occurs typically within a few min of illumination, during which time photosynthetic rates are still rather low (18). However, adjustments in photosynthetic metabolite pools (e.g. increase in PGA,[2] RuBP) are generally more prolonged and maximum photosynthetic rates are not observed until the active metabolite pools are established (11, 17, 18).

Induction in C_4 species is rather complicated since establishing maximum photosynthetic rates requires a coordinated functioning between the RPP pathway and the C_4 cycle. In leaves of the C_4 species maize, a buildup of RPP pathway intermediates and other organic-P, as well as adjustments in C_4 cycle metabolites, occurs during the induction period, particularly during the first 10 min of illumination (11, 17). The fact that isolated maize mesophyll protoplasts (which lack the RPP pathway) also show a buildup of triose-P during illumination has led, in part, to the suggestion (6) that during induction there may be an exchange of carbon between the C_4 pathway and the RPP pathway via PEP→triose-P (which would be independent of C_4 acid decarboxylation). Furthermore, there is evidence that an unknown carbon source from dark reserves may support buildup of photosynthetic pools during the induction period in maize (17).

The C_4 cycle is not autocatalytic, although carbon flow from PGA of the RPP pathway to three carbon intermediates of the C_4 cycle may be possible in some cases. The C_4 cycle operates with transport of C_4 acids and C_3 metabolites between the leaf mesophyll and bundle sheath cells. If intercellular transport occurs by symplastic diffusion, then metabolite concentration gradients of about 10 mM are calculated to be required to mediate sufficient carbon flux to support the observed rates of photosynthesis (9). Recent techniques for differential extraction of maize mesophyll and bundle sheath metabolites provide evidence of such gradients (e.g. malate), which are present in illuminated, but not in darkened, leaves (15).

There remains much uncertainty about the formative nature of intercellular metabolite gradients and even about the particular metabolic events associated with induction in C_4 species. The investigation of $^{14}CO_2$ metabolism has allowed the understanding of many aspects of photosynthesis. However, to date there apparently have been no reports on the metabolism of $^{14}CO_2$ during induction in any C_4 species. Here we examine the initial products of $^{14}CO_2$ assimilation and whole leaf metabolite contents of C_4 cycle intermediates during induction in the $NADP^+$-malic enzyme type C_4 dicot, *Flaveria trinervia*.

MATERIALS AND METHODS

Cellulose MN 300 was from Brinkmann Instruments Co. X-ray film (X-OMAT AR-5) and sec-butyl alcohol were from Eastman Kodak Co. $NaH^{14}CO_3$ (55.9 mCi/mmol) was from ICN Biomedicals, Inc. Glutamate dehydrogenase was from Boehringer Mannheim Biochemicals. Other commercial enzymes and most substrates were from Sigma Chemical Co.

Plant Material. Plants of *Flaveria trinervia* were maintained in a growth chamber with a 27/22°C day/night thermoperiod, a 14 h photoperiod, and an irradiance of 400 $\mu E/m^2 \cdot s$ at plant height. Plants were grown in perlite and watered daily to runoff with a nutrient solution of standard Hoagland composition (+30 mM NaCl [pH 5.9–6.1]), but with N provided as follows: high N treatment (15 mM NO_3^-, 2 mM NH_4^+), low N treatment (0.6 mM NO_3^-, 0.08 mM NH_4^+), and ammonia treatment (3.4 mM NH_4^+). In the ammonia treatment, plants were grown initially under high N and then under ammonia for 10 to 12 d before the

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[2] Abbreviations: PGA, 3-phosphoglycerate; RuBP, ribulose 1,5-bisphosphate; RPP pathway, reductive pentose phosphate pathway; PEP, phosphoenolpyruvate; MDH, malate dehydrogenase; LDH, lactate dehydrogenase.

experiments (seedlings grew poorly if provided initially with NH_4^+ alone). To standardize leaf material for differential aging effects induced by the varied N treatments, plants were allowed to grow to peak vegetative maturity prior to experiments. *F. trinervia* is an annual composite with a determinate flowering mode and fairly consistent growth patterns. Experiments were carried out on fully expanded leaves of the eighth stem node.

$^{14}CO_2$ Leaf Exposures and Identification of ^{14}C-Products. Plants were taken at 4 to 6 h into the light period and dark treated for 30 min. Leaves were excised, placed in a 36 ml glass vial with the petiole in distilled H_2O, and flushed with humidified air for 2 min (340 μl/L CO_2). In the standard procedure, leaves were illuminated (1000 $\mu E/m^2 \cdot s$ from three 150-W Westinghouse PAR lamps) for indicated times under normal air at room temperature (~25°C), then the vial was flushed 10 s with humidified, CO_2-free air and was quickly sealed with a serum stopper. $^{14}CO_2$ was injected (390 μl/L) for 10 s exposures. Injection of a smoke tracer into a similarly sealed vial confirmed that the injected gas was completely dispersed in less than 0.5 s. After $^{14}CO_2$ exposures, leaves were quickly killed by plunging into boiling 80% (v/v) ethanol. About 25 to 28 s elapsed from the initial flushing with CO_2-free air to killing of the leaf. Leaves were extracted as per Rumpho *et al.* (13) and the radioactivity in the final insoluble fraction was less than 3% of the total ^{14}C incorporated. Typically, about 8 to 10% of the available $^{14}CO_2$ was incorporated by the leaf. In no case was the $^{14}CO_2$ concentration depleted to less than 320 μl/L. Leaf extracts were partitioned with $CHCl_3$ and concentrated. Labeled products were separated and identified by two-dimensional thin-layer electrophoresis and chromatography on cellulose plates, followed by autoradiography (14). Recovery of radioactivity from the plates was > 90%. Glycerate was counted with PGA due to limited hydrolysis of phosphate esters in boiling ethanol (1).

Leaf Extraction for Metabolite Measurements. Leaves were dark adapted and illuminated as above, and then killed at the indicated times in liquid N_2. About 0.2 g of material (weights recorded) were extracted with frozen $HClO_4$ (0.6 N in about 1.3 ml final volume) essentially as described by Leegood and Furbank (11). To the extract, 20 μl of 875 mM Mes was added, and the pH then carefully adjusted to pH 6.5 with concentrated KOH. Following decolorization with activated charcoal (20 mg), extracts were centrifuged and utilized immediately for metabolite assays. Leaf subsamples ($n = 4$ or 5) were weighed and extracted with 96% ethanol (final volume) and total Chl determined according to Wintermans and De Mots (19). Metabolite contents were then expressed per mg Chl.

Metabolite Measurements. All assays were done in a spectrophotometer in a volume of 1 ml and were rigorously tested for proper reaction conditions and reaction end-points within a prescribed range of metabolite amounts. Malate was measured as per Gutmann and Wahlefeld (7). Glutamate was measured in 250 mM Tris-acetate (pH 8.7), 2 mM NAD, and 4 units of glutamate dehydrogenase. Aspartate and 2-oxoglutarate were determined by a coupled assay in similar reaction media containing 50 mM Hepes (pH 7.5), 30 μM pyridoxal phosphate, 0.3 mM NADH, 1 unit MDH, and either 1 unit aspartate aminotransferase and 2.5 mM 2-oxoglutarate (for aspartate) or 0.5 unit aspartate aminotransferase and 2.5 mM aspartate (for 2-oxoglutarate). Pyruvate and PEP were determined sequentially by a coupled assay in media containing 90 mM Tes-Tris (pH 7.5), 42 mM KCl, 12 mM $MgCl_2$, 2 mM ADP, 0.3 mM NADH, and 1 unit LDH. After the pyruvate measurement, 0.5 unit pyruvate kinase was added to assay for PEP. PGA was assayed in a coupled system containing 90 mM Tes-Tris (pH 7.5), 42 mM KCl, 12 mM $MgCl_2$, 0.3 mM NADH, 1 mM PEP, 5 mM ATP, 2 units glyceraldehyde 3-P dehydrogenase, 1 unit pyruvate kinase, and 3 units PGA kinase (used to initiate the reaction). In this system ADP is recycled to ATP, which allowed the end-point to be reached more quickly. Alanine was assayed in a coupled system containing 100 mM Tris-HCl (pH 8.0), 2.5 mM 2-oxoglutarate, 5 mM $MgCl_2$, 2 mM EDTA, 0.3 mM NADH, 30 μM pyridoxal phosphate, 1 unit LDH, and 3 units alanine aminotransferase. A known amount of alanine was included in each group of assays and aliquots were gauged to keep the change in $A_{340} < 0.3$.

Enzyme Assay. Leaves were powdered with liquid N_2 in a mortar and pestle, and were ground in 4 volumes of medium containing 100 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM EDTA, 3 mM DTE, 80 μM pyridoxal phosphate, 25% (w/v) insoluble PVP, and 0.05% Triton X-100. Following filtration and centrifugation, extracts were assayed directly for alanine (:2-oxoglutarate) aminotransferase activity (EC 2.6.1.2) as described by Edwards and Gutierrez (3).

Interveinal Measurements. Interveinal distances were measured in leaves cleared with 5% NaOH, dehydrated with ethanol, stained 6 min with 0.1 M $KMnO_4$, and mounted on slides. Veins were traced from image projections, total vein length (mm/4 mm^2) determined with a calibrated planimeter, and interveinal distances then calculated.

RESULTS

During induction, the rate of $^{14}CO_2$ incorporation by leaves of *Flaveria trinervia* was biphasic, with an initial rapid increase through the first min of illumination, followed by a more gradual increase during the remainder of the 20 min illumination period (12). Preliminary experiments of $^{14}CO_2$ leaf exposures showed distinctive changes in the relative labeling of aspartate and malate, with a transient increase in aspartate labeling observed after 5 min of illumination (e.g. Fig. 1). To maximize the amount of label incorporated at each exposure and to maintain a near ambient CO_2 concentration during the pulse, our standard procedure included a 10 s flush of the labeling vial with humidified CO_2-free air just prior to leaf exposures to $^{14}CO_2$. This prevented dilution of the label during each exposure, but did not affect the

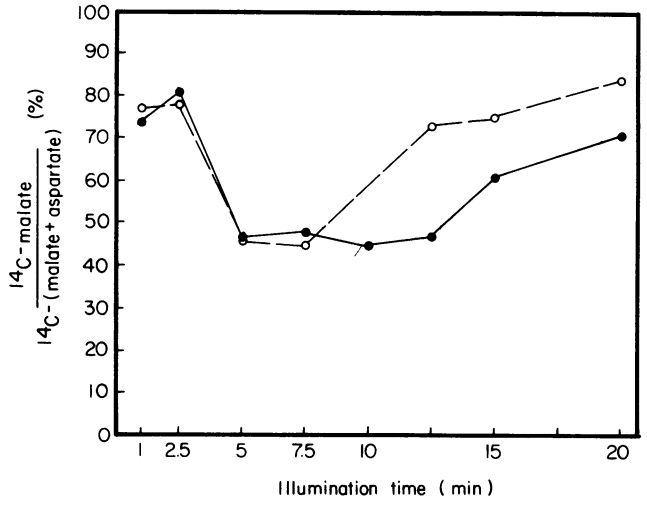

FIG. 1. Percent of [^{14}C]malate relative to [^{14}C](malate + aspartate) formed during induction in *F. trinervia* after varying *in vitro* conditions prior to labeling with $^{14}CO_2$ (plants grown with high N levels). During illumination, the labeling vial with the leaf was flushed continuously with humidified ambient air (about 340 μl CO_2/L of air by IR gas analyzer). At the indicated times the vial was flushed 10 s with humidified CO_2-free air (●——● control) or continued flushing with $^{12}CO_2$-containing air (O- - -O continuous CO_2) prior to exposure to $^{14}CO_2$ (final amount of CO_2 about 390 μl/L in each case). See "Materials and Methods" for details on the killing procedure, the separation of products, and autoradiography.

basic labeling patterns observed throughout this study. Whether leaves were labeled after a 10 s flush with CO_2-free air or after constant exposure to CO_2-containing air, a similar transient decrease in the relative labeling of malate was observed after 5 min of illumination (Fig. 1). The partitioning of label between malate and aspartate is essentially constant for varying pulse times of 3 to 12 s in this C_4 species (B d Moore, unpublished data).

Labeling patterns from leaf $^{14}CO_2$ exposures during induction with high N grown plants showed several prominent features (Fig. 2). During the first min of illumination, malate was the predominant product with 77% of the total label (increasing only slightly in this period), while asparate had only about 18% of the initial label. However, beginning 2.5 min after illumination, there was a dramatic shift in label appearing more in aspartate, with an apparent peak at about 5 min (66% of soluble ^{14}C-products). After this time, there was a gradual decline in the proportion of ^{14}C to aspartate and concomitant increase in the percent ^{14}C to malate. Other metabolites contained at most 15% of the initial label at any time during the illumination period, a pattern consistent with expression of C_4 photosynthesis after a 10 s pulse of $^{14}CO_2$.

The relative labeling of aspartate *versus* malate could not be accounted for by changes in the apparent activity of NADP-MDH, which showed a rapid 7-fold increase in activity to maximum levels after 2 min of illumination (B d Moore, unpublished data). Clearly, the relative formation of [^{14}C]malate was not restricted even after only 15 s of illumination (while the activity of NADP-MDH was still rather low).

To further investigate the nature of the observed labeling patterns we sought to perturb the plant's metabolic status by varying the N nutrition. A previous study of C_4 grasses (16) indicated that in some species, nitrogen nutrition may influence the partitioning of carbon between malate and aspartate. For example, maize when treated with ammonia as sole N source (*versus* NO_3^-) showed a 15% increase in the proportion of aspartate as a primary product and a similar decrease in percentage labeling of malate (16). Also, increasing amounts of nitrate N during growth of maize plants is associated with a large decrease in the leaf aspartate content (2).

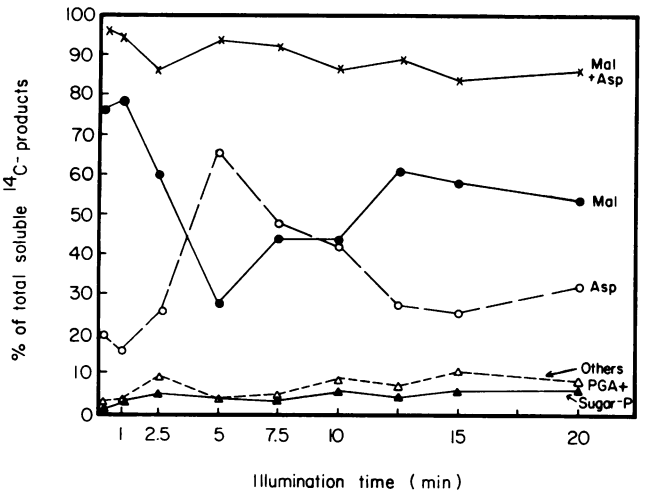

FIG. 2. Initial products of $^{14}CO_2$ assmilation during photosynthetic induction in *F. trinervia* grown with high N levels (17 mM). At the indicated times the labeling vial with the leaf was flushed 10 s with humidified CO_2-free air, sealed, and exposed to $^{14}CO_2$ (390 μl/L) for 10 s. See "Materials and Methods" for further details. The amount of ^{14}C incorporated after 10 s pulses at different times of illumination ranged from 87,000 to 251,000 dpm cm^{-2}. The respective major ^{14}C-products are indicated in the figure. Abbreviations are as follows: mal, malate; asp, aspartate; PGA; and sugar-P, sugar phosphates.

Similar labeling experiments were carried out during illumination of leaves of *F. trinervia* grown under limiting N (0.68 mM) or grown 10 to 12 d with ammonia as the N source (see "Materials and Methods"). In both cases, in the first min of illumination malate was the predominant initial product during 10 s pulses with $^{14}CO_2$ (about 75% at 1 min), and a subsequent surge in label to aspartate was observed (Figs. 3 and 4). In plants grown with low N, this increase in label to aspartate was delayed to about 7.5 min, showing a maximum increase of 37%. Prior treatment of plants with ammonia did not alter the timing of the surge of label to aspartate from that observed with high N grown plants, but did diminish the magnitude of the increase to about 22%. The ammonia treatment was toxic if too prolonged, but did effectively abolish nitrate reductase activity, without visible damage (observations). In a replicate set of pulse experiments, the described labeling patterns were consistently observed with

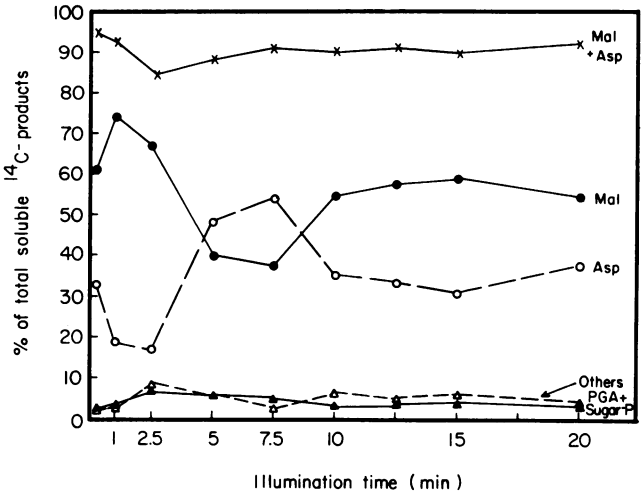

FIG. 3. Initial products of $^{14}CO_2$ assimilation during photosynthetic induction in *F. trinervia* grown with low N levels (0.68 mM). See Figure 2 or "Materials and Methods" for further details. The amount of ^{14}C incorporated after 10 s pulses at different times of illumination ranged from 164,000 to 503,000 dpm cm^{-2}.

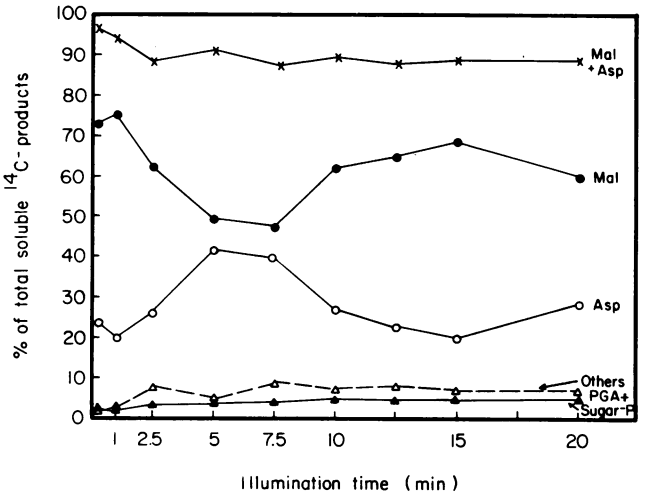

FIG. 4. Initial products of $^{14}CO_2$ assimilation during photosynthetic induction in *F. trinervia* grown with ammonia (3.4 mM) as the N source for 10 to 12 d prior to the experiment. See Figure 2 or "Materials and Methods for further details. The amount of ^{14}C incorporated after 10 s pulses at different times of illumination ranges from 196,000 to 543,000 dpm cm^{-2}.

plants of the various N treatments (data not presented).

To gain further insight into C_4 metabolism during induction, C_4 metabolites in high and low N grown plants were determined from perchlorate extracts of whole leaves treated as above. For all metabolites there was good recovery (greater than 84%) of internal standards when added at the start of extraction procedures (Table I).

During the first min of illumination, leaves of high N plants showed a large, temporary decrease in pyruvate and a transient increase in alanine (the latter from 860–1000 nmol/mg Chl, Fig. 5). Between 1 and 20 min illumination time there was a marked decrease in alanine (from 1000–180 nmol/mg Chl), while pyruvate levels stabilized by about 7.5 min. Levels of both aspartate and malate declined substantially between 1 and 2.5 min of illumination, with malate showing a subsequent gradual increase thereafter. Aspartate underwent a sudden doubling of pool size (to 395 nmol/mg Chl) between 5 and 7.5 min before gradually declining to 195 nmol/mg Chl after 20 min illumination. While the maximum levels of both PEP and PGA were less than 90 nmol/mg Chl, each increased significantly following illumination.

Changes in the C_4 metabolites of plants grown with low N were in many respects similar to those observed above (Fig. 6). Notably, the decrease in pyruvate during the first min was less pronounced, but the initial transient increase in alanine was of greater magitude (from 295–630 nmol/mg Chl) and its peak was shifted to 2.5 min, before declining. Malate showed an initial decrease and then a sudden increase between 1 and 2.5 min, remaining somewhat constant thereafter. Similar to before, aspartate showed an initial transient decrease, then a sudden 2-fold increase between 5 and 10 min (to 220 nmol/mg Chl), but afterwards remaining constant.

In plants of both growth conditions, the maximum fluctuations in malate during illumination were quite large (2.6 and 1.5 μmol/mg Chl, low and high N plants, respectively). In both cases there were very similar patterns in the change in malate levels and that observed in total C_4 cycle metabolites (malate + aspartate + PEP + pyruvate + alanine).

The rather striking transient increases in leaf alanine contents (per mg Chl) under different N treatments, were evaluated relative to leaf area, interveinal distance, and alanine aminotransferase activity since these factors likely influence the potential for intercellular fluxes. As leaf Chl content ($\mu g/cm^2$) was highest in plants grown with high N, maximum alanine contents on a leaf area basis showed even greater differences (3.5-fold) between plants grown with high *versus* low N (Table II). Similarly, alanine aminotransferase was considerably more active on a leaf area basis in plants grown with high N (although activities on a Chl basis were rather similar, 460–520 μmol/h mg Chl). However, there was a minimal effect of N nutrition on interveinal distances, even though leaf areas were reduced about 45% in plants grown with low N (Table II).

Table I. *Recoveries of Metabolites from Whole Leaves of F. trinervia*

Leaves were cut, placed in liquid N_2, powdered in a mortar and pestle, and divided into six portions of about 0.2 g each. Three subsamples were extracted in $HClO_4$ and metabolites measured (control values, nmol/g fresh wt). For the other three subsamples, aliquots of concentrated, known metabolite stocks were added to the mortar and pestle prior to extraction. Added nmol levels of metabolites were varied to give amounts about equal to the respective endogenous levels previously determined in other samples.

Metabolite	Recovery
	% ± SD
Malate	97 ± (9)
Aspartate	90 ± (5)
2-Oxoglutarate	95 ± (5)
Glutamate	100 ± (3)
PGA	84 ± (1)
Pyruvate	92 ± (6)
PEP	103 ± (7)
Alanine	89 ± (9)

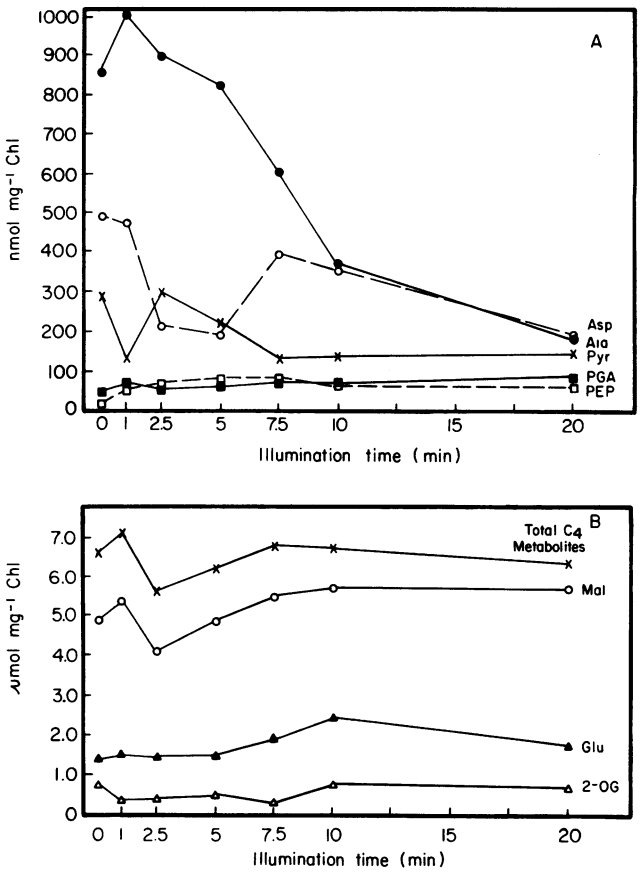

FIG. 5. Leaf metabolite contents during photosynthetic induction in *F. trinervia* grown with high N levels (17 mM). To approximate conditions used for labeling, leaves were excised and placed in the labeling vial during illumination. At the indicated times, vials were flushed 10 s with humidified CO_2-free air and leaves then quickly plunged into liquid N_2. Leaves were stored in liquid N_2 prior to perchlorate extractions and metabolite assays. Standard errors (n = 3 or 4) averaged 8.1% of the indicated mean values. Respective metabolites are indicated in the figure. Abbreviations are as in Figure 2, plus the following: ala, alanine; pyr, pyruvate; PEP, phosphoenolpyruvate; glu, glutamate; and 2-OG, 2-oxoglutarate. Total C_4 metabolites equals the sum of the measured metabolites of the C_4 cycle (mal + asp + pyr + PEP + ala).

DISCUSSION

During induction, labeling patterns from $^{14}CO_2$ pulses to whole leaves of *F. trinervia* showed three distinct phases (Figs. 1–4): (a) During the first min, malate was the predominant C_4 acid labeled (62–76% at 15 s), and the percentage label in malate increased only slightly during this time (to 74–78%); (b) a transient increase in the proportion of label to aspartate occurred after the initial phase at about 5 min in both high N and ammonia treated plants. This surge in proportioning of label to aspartate was delayed to about 7.5 min in low N plants; and (c) during later times, label to malate was again predominant, although sometimes containing a lower percentage labeling after 20 min than after 1 min of illumination.

In general, the partitioning of fixed $^{14}CO_2$ between malate and

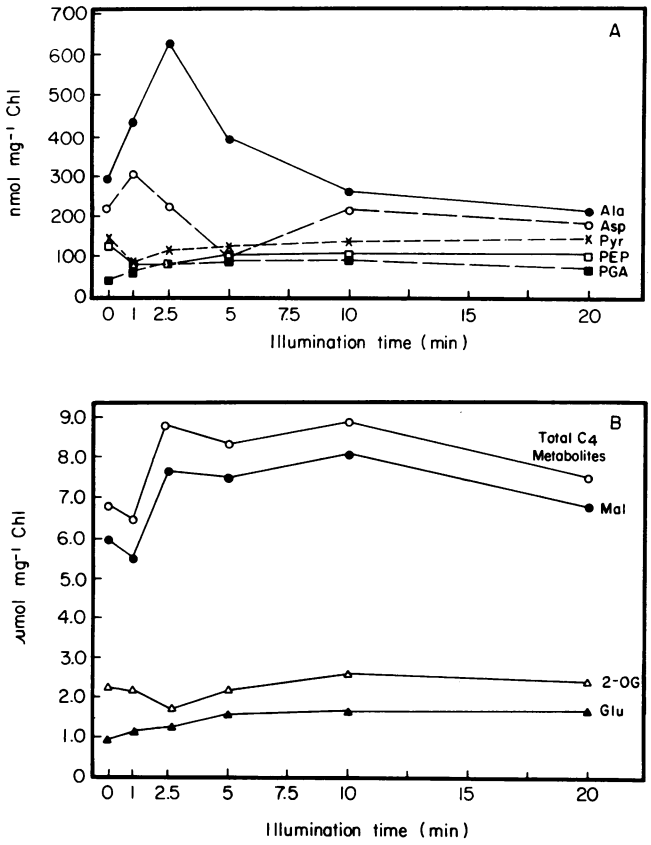

FIG. 6. Leaf metabolite contents during photosynthetic induction in *F. trinervia* grown with low N levels (0.68 mM). See Figure 5 for further details. Standard errors ($n = 3$ or 4) averaged 7.2% of the indicated mean values.

aspartate during the pulse treatments reflects the relative rate of synthesis of each metabolite in the mesophyll cells. For photosynthesis to reach maximum rates in C_4 plants it is considered that sufficient intercellular gradients of metabolites need to develop including high concentrations of malate and aspartate in mesophyll cells. Whether these gradients are established from dark carbon reserves or carbon assimilation during induction is yet to be established. However, due to oscillations in carbon input to malate and aspartate from CO_2 fixation during induction, it is likely that the active pools of these C_4 acids in mesophyll cells undergo changes until steady state photosynthesis is reached.

Fluctuations in leaf levels of C_4 metabolites (Figs. 5 and 6) indicate several likely metabolic events during induction. During the first min of illumination, a sudden decrease in pyruvate content was observed, similar to that described in maize (11, 17). This likely reflects pyruvate uptake and metabolism by the mesophyll chloroplast. A recent demonstration of a rapid, light-dependent uptake of pyruvate by maize mesophyll chloroplasts (5) is thought to be important for establishing a sink in the mesophyll cells for diffusion of pyruvate/alanine from the bundle sheath cells.

Another early response during induction is the large decrease in total leaf malate content. Similar results have been observed with maize, but the magnitude of decline in the level of malate in *F. trinervia* (1.6 μmol/mg Chl) is much larger than that reported in maize (0.2–0.4 μmol/mg Chl; 11, 17). This response is suggestive of an initial, extensive malate decarboxylation in the bundle sheath chloroplast, but the basis for the light requirement of such a putative event is of unknown nature. However, such a response could facilitate formation of a malate diffusion gradient from mesophyll to bundle sheath cells and may also contribute directly to a buildup of RPP pathway intermediates.

Along with transient changes in aspartate labeling during induction, there were fluctuations in the aspartate content of the leaf. After the surge in aspartate labeling at 5 min, leaf aspartate content doubled by 7.5 min (high N plants). In one study with maize, there was a comparable increase (although between 1 and 3 min of illumination) in total leaf aspartate, which followed an initial decrease in plants dark treated for 20 min (17). Aspartate is probably formed with glutamate as the amino donor. Leaf glutamate content gradually increased during induction (Figs. 5 and 6), but showed no obvious relationship to the observed transient increase in aspartate labeling or in total leaf aspartate content. Also, altered glutamate metabolism due to different activities of NO_3^- assimilating enzymes is not of primary importance since the ammonia treated plants had no NO_3^- reductase activity.

Of particular interest here are the early transient increases observed in leaf alanine contents and their subsequent gradual decline observed during the remainder of the induction period. The increases were of smaller magnitude in *F. trinervia* (140 and 335 nmol/mg Chl, high and low N plants, respectively) than that observed in maize by Usuda (increase after 30 s of 700–800 nmol/mg Chl, Ref. 17). Plants grown here with limiting N displayed a delay in this peak from 1 min (high N grown) to about 2.5 min. There is evidence from pulse/chase experiments of a major photosynthetically active alanine pool in *F. trinervia* even very early during induction, which likely becomes smaller and turns over more rapidly as the metabolic conditions approach steady state (12). In plants of both high and low N treatments, as alanine pools decreased to near a steady state level, malate again became the predominant initial photosynthetic product (Figs. 2, 3, 5, and 6).

Previous experiments with mesophyll extracts of *Digitaria*

Table II. *Influence of Nitrogen Nutrition on Selected Leaf Parameters of F. trinervia*

Plants were grown in perlite and given daily nutrients with varying N as follows: high N, low N, and ammonia (see "Materials and Methods"). Alanine contents were calculated from maximum amounts observed during illumination. Interveinal distances were calculated from measured vein lengths of image projections of fully expanded leaves which had been cleared in 5% NaOH and stained 6 min with 0.1 M $KMnO_4$. Distances are averages of values determined from two different areas on each of three leaves. The total range in calculated interveinal distances from plants grown with 0.14 to 85 mM total N (NO_3^- + NH_4^+) was 106 to 126 μm.

Nitrogen Treatment	Chl Content	Maximum Alanine Content	Alanine Aminotransferase Activity	Average Interveinal Distance	Average Leaf Area
	$\mu g/cm^2$	$nmol/cm^2$	$\mu mol/h \cdot cm^2$	μm	cm^2
High	71	71	35.6	124	23.2
Low	30	19	13.9	112	12.7
Ammonia	46	28	23.9		21.5

sanguinalis (generally a 'malate former') showed aspartate to be the preferred product in the presence of alanine and 2-oxoglutarate (10). Perhaps the transient increase in aspartate labeling observed during induction in *F. trinervia* may occur through a surge in the availability of alanine to the C_4 cycle, with different N treatments possibly affecting this latter, suggested event. High N grown plants of *F. trinervia* had substantially greater maximum leaf alanine content and alanine aminotransferase activity (on a leaf area basis) than did plants of either low N or ammonia treatments (Table II). The high N grown plants also showed the largest transient increase in aspartate labeling (Figs. 2–4). That high N grown plants displayed an earlier transient peak in leaf alanine content relative to low N grown plants, would suggest that the former plants were able to more rapidly form the active leaf alanine pool. This might account for the delayed surge in label to aspartate in the low N grown plants. The ammonia treated plants did not show a similar delay in the timing of the increased aspartate labeling, but they also did not show a similarly delayed transient peak in leaf alanine content (B d Moore, unpublished data).

We would like to point out that PGA levels are very low in *F. trinervia* relative to other C_4 species (85 nmol/mg Chl *versus* 340–450 nmol/mg Chl in maize, [15; 17; B d Moore, unpublished data); and 880 nmol/mg Chl in *Chloris*, [8]), but are approximately the same as commonly observed in many C_3 species (e.g. 4). Since maize is PSII deficient in the bundle sheath chloroplast, up to one-half of the PGA from RuBP carboxylase activity is suggested to return to the mesophyll for reduction to triose P (4). The measured PGA amounts in the two cell types of maize are considered sufficient to account for its diffusion from bundle sheath to mesophyll (15). A similar situation appears unlikely in *F. trinervia* due to the low PGA levels and evidence that this species does have appreciable noncyclic electron transport capacity in its bundle sheath chloroplasts (>70 μmol O_2/h mg Chl; B d Moore, unpublished data).

In *F. trinervia*, pyruvate and alanine presumably are the major three carbon metabolites that diffuse from the bundle sheath to the mesophyll; however, their steady state levels were only about 150 to 220 nmol/mg Chl (Figs. 5 and 6). Neither pyruvate nor alanine appear to be present in sufficient amounts to each form a 10 mM intercellular gradient (e.g., 9, 15), although somewhat smaller gradients may still support carbon flux through the C_4 cycle if both metabolites contribute to PEP formation in the mesophyll cells.

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