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

II. METABOLISM OF PRODUCTS OF $^{14}CO_2$ FIXATION AFTER DIFFERENT ILLUMINATION TIMES

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ABSTRACT

The metabolism of fixed $^{14}CO_2$ and the utilization of the C-4 carboxyl of malate and aspartate were examined during photosynthetic induction in *Flaveria trinervia*, a C_4 dicot of the NADP-malic enzyme subgroup. Pulse/chase experiments indicated that both malate and aspartate appeared to function directly in the C_4 cycle at all times during the induction period (examined after 30 seconds, 5 minutes and 20 minutes illumination). However, the rate of loss of ^{14}C-label from the C-4 position of malate plus aspartate was relatively slow after 30 seconds of illumination, compared to treatments after 5 or 20 minutes of illumination. Similarly, the appearance of label in other photosynthetic products (e.g. 3-phosphoglycerate, sugar phosphates, alanine) during the chase periods was generally slower after only 30 seconds of leaf illumination, compared to that after 5 of 20 minutes illumination. This may be due to the lower rate of photosynthesis after 30 seconds illumination. The appearance of label in carbons 1→3 of each C_4 acid during the chase periods was relatively slow after either 30 seconds or 5 minutes illumination, while there was a relatively rapid accumulation of label in carbons 1→3 of both C_4 acids after 20 minutes illumination. Thus, while the turnover rate of the ^{14}C-4 label in both C_4 acids increased only during the first 5 minutes of the induction period, only later during induction is there an increased rate of appearance of label in other carbon atoms of the C_4 acids. The implied source of ^{14}C for labeling of the 1→3 positions of the C_4 acids is an apparent carbon flux from 3-phosphoglycerate of the reductive pentose phosphate pathway to phosphoenolpyruvate of the C_4 cycle.

C_4 photosynthesis operates via biochemical specializations of leaf mesophyll and bundle sheath cells, in which products of PEP[2] carboxylase in the mesophyll (ultimately malate and/or aspartate) serve as donors of CO_2 to the RPP pathway in the bundle sheath. C_4 plants undergo metabolic responses during photosynthetic induction which likely lead to the formation of the intercellular metabolite gradients which are considered necessary for carbon flux through the C_4 cycle (e.g. 5, 13). Recently, Stitt and Heldt (12) were able to detect gradients of several transport metabolites between mesophyll and bundle sheath cells of maize, following differential filtration of previously illuminated leaf material ground in liquid N_2.

There is considerable uncertainty about the interactions between the C_4 cycle and the RPP pathway in C_4 plants as metabolite pools become established during induction. Shieh *et al.* (11) suggested that dark reserves of aspartate in the bundle sheath cells of crabgrass may be an endogenous source of CO_2 early during illumination. Such metabolism could also contribute to a buildup of pyruvate/alanine in the bundle sheath cells, and help establish the gradient for return of a three carbon acceptor to the mesophyll cells. Another type of interaction includes possible carbon exchange between triose-P/PGA and PEP (1, 4). In the mesophyll cells this may show directional flexibility according to the expression of PEP carboxylase activity (e.g. 12) and likely, of pyruvate, Pi dikinase activity. There is some evidence that the particular 'balance' between the rate of C_4 acid production and the availability of three carbon precursors to PEP carboxylase may be slow to develop during induction. A large transient increase in the relative partitioning of $^{14}CO_2$ to aspartate (*versus* malate) was observed in the C_4 dicot *Flaveria trinervia* after 5 min illumination, suggesting that such transient high amounts of aspartate are formed as in an 'overflow' mechanism (7).

In the present study, $^{14}CO_2$ pulse/chase experiments were conducted after three different illumination times of dark-adapted plants of *F. trinervia* (NADP-malic enzyme subgroup). The selected illumination times (30 s, 5 min, and 20 min) correspond to different metabolic phases according to previously observed $^{14}CO_2$ partitioning patterns (7). In particular, we wanted to evaluate the utilization of malate and aspartate formed at different times during induction by examining the turnover and subsequent fate of label from the C-4 position of the two C_4 acids.

MATERIALS AND METHODS

Cellulose MN 300 was from Brinkmann Instruments Co. X-ray film (X-OMAT AR-5) and 2-butanol were from Eastman Kodak Co. $NaH^{14}CO_3$ (55.9 mCi/mmol) was from ICN Biomedicals, Inc. [U-^{14}C]malate and [4-^{14}C]aspartate were from Amersham. Chicken liver malic enzyme and porcine heart glutamate-oxaloacetate transaminase were from Sigma Chemical Co.

Plant Material. Plants of *Flaveria trinervia* were grown from seed and maintained in a growth chamber as previously described (6). 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]). Total N was 17 mM (15:2, NO_3^-:NH_3). Experiments were done with leaves of the eighth node, at full expansion.

$^{14}CO_2$ Leaf Exposures and Identification of ^{14}C-Products. Plants were dark adapted 30 min and leaves were exposed to $^{14}CO_2$ (390 μl/L) for 10 s (after a 10 s flush of the labeling vial with CO_2-free air) as previously described (7). In the chase experiments, leaves were transferred from the labeling vial to an adjacent, illuminated beaker (1000 $\mu E\ m^{-2} \cdot s^{-1}$) with the petioles kept in water. Humidified ambient air containing 340 μl/L $^{12}CO_2$

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[2] Abbreviations: PEP, phosphoenolpyruvate; RPP pathway, reductive pentose phosphate pathway; PGA, 3-phosphoglycerate; $t_{½}$, turnover half-time.

was continuously passed over the leaf surfaces prior to killing at indicated times in boiling 80% (v/v) ethanol. Leaf extractions and identification of ^{14}C-products were as previously described (9, 10). Glycolate authenticity was verified by the relative migration of known [^{14}C]glycolate on TLC plates and by HPLC of the isolated metabolite (Bd Moore, unpublished data).

Partial Degradation of Isolated Malate and Aspartate. Malate and aspartate were isolated as above (TLE/TLC) and analysis of the percent label in the C-4 carboxyl group of each was carried out essentially as described by Hatch (3). However, in this study $^{14}CO_2$ released via catalysis by chicken liver malic enzyme was trapped in a closed system on blotter paper wicks to which a controlled amount of 10% (v/v) ethanolamine had been added. Aspartate was converted to malate using aspartate aminotransferase and NAD malate dehydrogenase. Total malate concentration in each sample was determined from aliquots, using NAD malate dehydrogenase (2). Varying amounts of cold malate then were added to sample aliquots to attain 0.3 mM in each degradation vial. Total aspartate concentration in each sample was found to be negligible and cold aspartate was added to 0.3 mM.

Degradation of known standards of [U-^{14}C]malate and [4-^{14}C] aspartate resulted in about 95% recovery of label from the C-4 position after an 8 h incubation. These experiments also confirmed an absolute specificity by chicken liver malic enzyme (and malic enzyme from *F. trinervia*) for release of CO_2 from the C-4 carboxyl group of malate (Bd Moore, unpublished data).

RESULTS

Following a 30 min dark treatment of plants of *F. trinervia*, excised leaves showed a biphasic response of CO_2 assimilation with increasing time of illumination (Fig. 1). The rate of $^{14}CO_2$ incorporation increased rapidly during the first min or so of illumination, then gradually leveled off. The incorporation rate was only slightly greater after 20 min of illumination than after 5 min of illumination. The relative partitioning of carbon, however, showed at least three phases. The rate of incorporation of label in malate was relatively high during the initial 2.5 min of illumination. There was a single oscillation with aspartate labeling reaching a maximum after 5 min illumination, and malate labeling again predominating after 12.5 min of illumination.

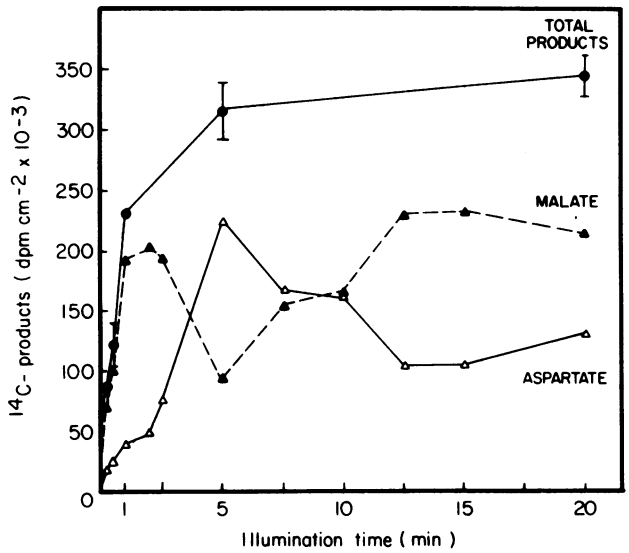

FIG. 1. Assimilation of $^{14}CO_2$ during a 10 s pulse after illumination of leaves from dark-adapted plants of *F. trinervia*. Leaves were exposed to $^{14}CO_2$ (390 μl/L, 10 s) and then immediately killed in boiling 80% ethanol. About 25 to 28 s elapsed from each illumination time prior to killing the leaf. Total ^{14}C incorporated includes both soluble and insoluble products (the latter being less than 3% of total). Values for 30 s, 5 min, and 20 min illumination times are means (SE) of 6 to 8 incorporations. Values for 15 s and 1 min illumination times are from Moore and Edwards (7). Relative rate of C_4 acid formation was calculated from the $^{14}CO_2$ incorporation rates multiplied by the respective relative partitioning of $^{14}CO_2$ between malate and aspartate (7).

Pulse/chase experiments with $^{14}CO_2$ were done after leaf illuminations for 30 s, 5 min, and 20 min. In all cases, the majority of the label was in the C_4 acids (malate plus aspartate) after the 10 s pulses, and the total label in the C_4 acids was observed to decrease during the 2 min chase period (Fig. 2). For the first 60 to 90 s of the chase, the rate of decrease in the total label was slowest in leaves which had received 30 s of illumination compared to leaves which had received either 5 or 20 min of illumination. In leaves illuminated for 20 min, an apparent, transient increase was observed in total label in the C_4 acids after a 60 s chase. In the latter part of the chase, the label in the C_4 acids remained higher in those leaves which had received 20 min of illumination compared to those which had received 5 min of illumination.

Partial degradation of each C_4 acid was done to clarify the labeling patterns observed in Figure 2. The rate of loss of label from the C-4 position of malate plus aspartate (relative to total label in other metabolites) was slowest in leaves which had been illuminated 30 s and was faster in those illuminated either 5 or 20 min (Fig. 3A). In either the 5 or 20 min illumination treatments, there was no apparent difference in the rate of loss of label from the C-4 position of the C_4 acids. A rapid loss of label from the C-4 of aspartate was observed following $^{14}CO_2$ pulses to leaves, particularly in those illuminated for 5 min (Fig. 3B). By comparison, the loss of label from the C-4 of malate showed an initial lag (Fig. 3C), but otherwise was much like the patterns observed for loss of label from both C_4 acids (Fig. 3A).

Data on the percentage specific labeling of the C-4 position of both malate and aspartate showed that the rate of loss of label from the C-4 position relative to the rate of appearance of label in carbons 1→3 was similar for each respective C_4 acid during the chase period in leaves which had been illuminated for 30 s or 5 min (Fig. 4, A and B). In comparison, in leaves which had received 20 min illumination, the specific labeling for the C-4

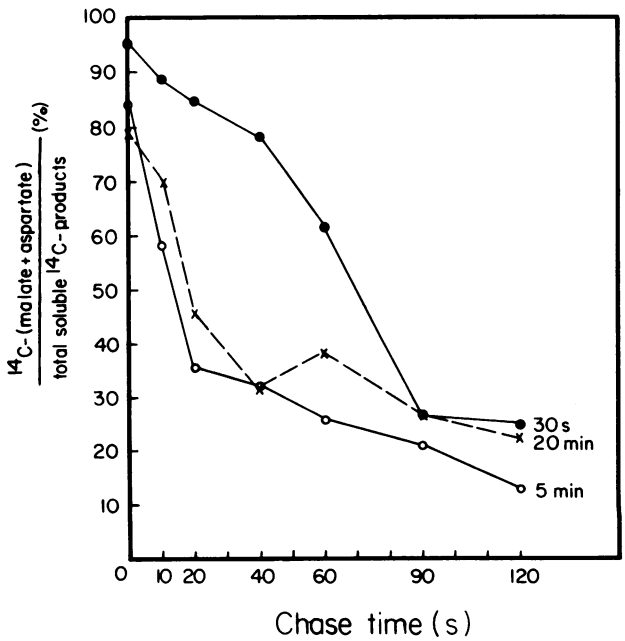

FIG. 2. Percent [^{14}C](malate + aspartate) of total soluble ^{14}C-products during chase periods after leaf illumination for 30 s, 5 min, and 20 min.

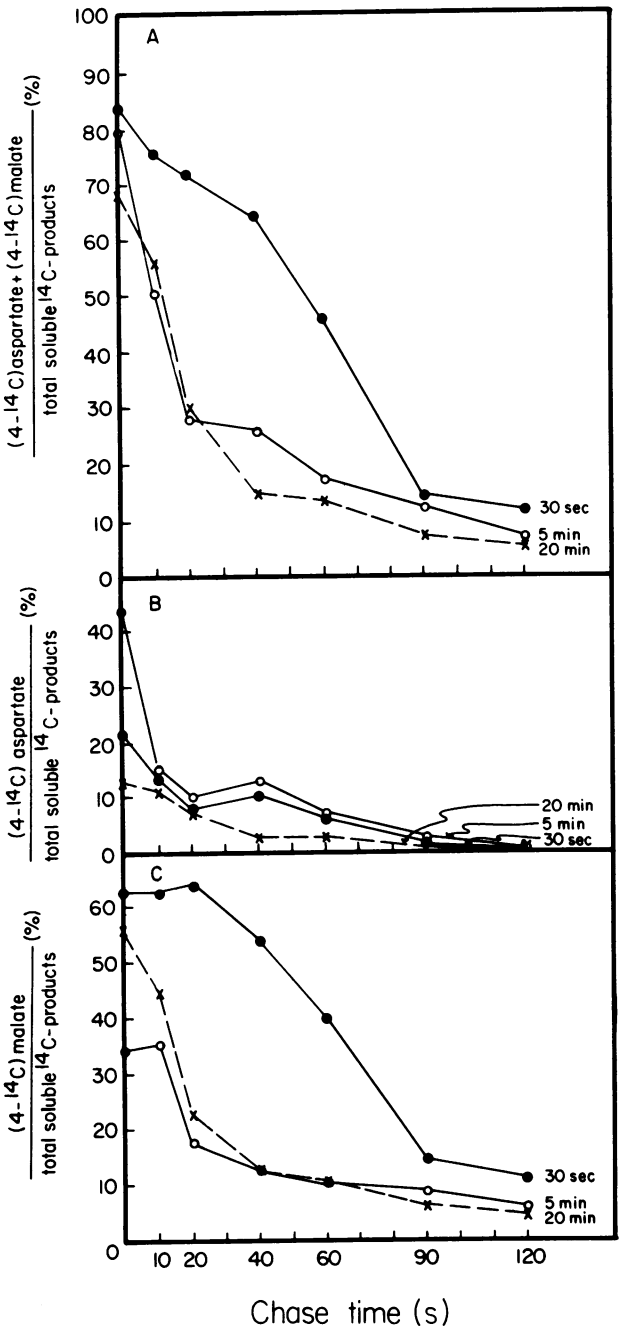

FIG. 3. A, Percent [4-^{14}C]malate + [4-^{14}C]aspartate relative to total soluble ^{14}C-products during chase periods following different times of leaf illumination. Values were calculated from data of Figures 2 and 4. B, [4-^{14}C]aspartate as a percentage of total labeled products during the chase periods. C, [4-^{14}C]malate as a percentage of total labeled products during the chase periods.

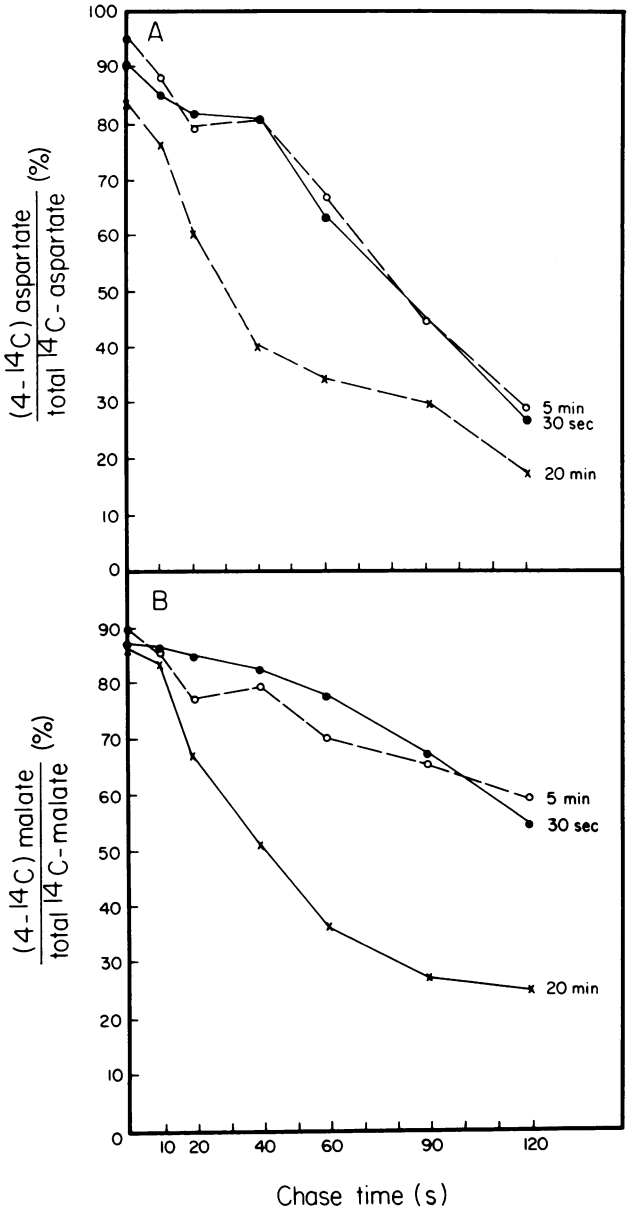

FIG. 4. A, Specific labeling of C-4 position of aspartate during chase periods after different illumination times. Isolated aspartate was converted to malate, with the carboxyl group at the C-4 position of malate released by malic enzyme and trapped with ethanolamine wicks. Values are means from two separate measurements and are expressed as percent ^{14}C-wick/(^{14}C-wick + ^{14}C-left in solution). B, Specific labeling of C-4 position of isolated malate.

compared to other positions of either malate or aspartate decreased much more rapidly.

The appearance of label in other products during the chase interval was also examined (Fig. 5). The maximum amount of label appearing in PGA was less than 7% during the 2 min chase period after pulses in leaves illuminated 30 s or 20 min, but was observed to increase to almost 18% in leaves which had received 5 min illumination. The rate of label appearing in sugar phosphates was considerably slower in leaves exposed to $^{14}CO_2$ after 30 s illumination (relative to after 5 or 20 min illumination). Formation of labeled succinate or citrate was most pronounced in those leaves illuminated for 30 s (majority of "others"). Substantial label appeared in alanine during the chase in all treatments, although the maximum amount of label (25%) occurred during the chase following the experiment with leaves which had received 30 s illumination. Notably, sucrose was formed in significant amounts after a 2 min chase in all treatments. However, in leaves illuminated only 30 s, there was a considerable lag (~1 min) before the eventual appearance of label in sucrose. Glycolate was also a major product, with an increased percentage labeling with longer illumination times (up to 18% during the chase after 20 min of illumination). Curiously about 9% of the total label was in glycolate after the initial 10 s pulse to leaves illuminated for 20 min.

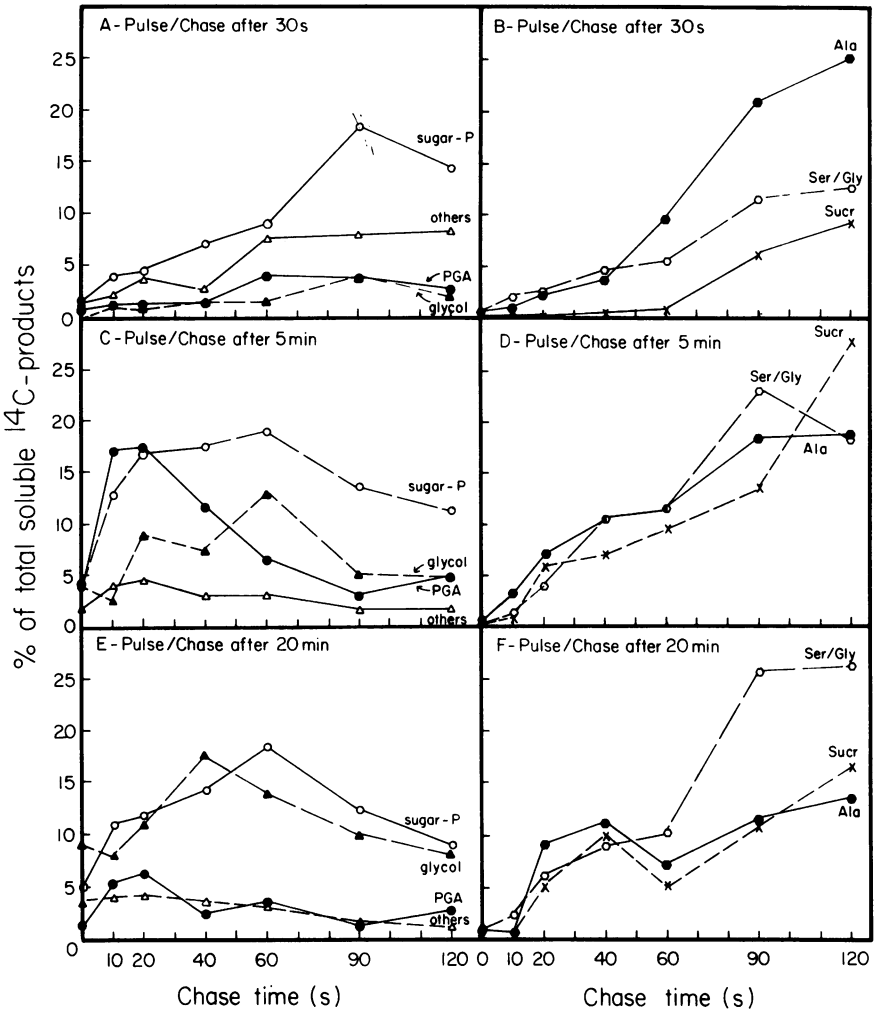

FIG. 5. Percent distribution of ^{14}C in photosynthetic products other than malate and aspartate during chase periods following the indicated leaf illumination times: (A, B), following 30 s illumination; (C, D), following 5 min illumination; (E, F), following 20 min illumination. Samples were from the same leaf exposures as those for the C_4 acids in Figure 2. Abbreviations are glycol, glycolate; ala, alanine; ser/gly, serine/glycine; sugar-P, sugar phosphates; sucr, sucrose.

DISCUSSION

Pulse/chase studies on $^{14}CO_2$ metabolism were conducted after three different times of illumination (30 s, 5 min, and 20 min) in dark-adapted leaves of *F. trinervia*. Rates of $^{14}CO_2$ incorporation in *F. trinervia* increased rapidly during the first min of illumination, then increased more gradually through the first 20 min of illumination (Fig. 1). A similar pattern of CO_2 assimilation (using IR gas analysis) has been observed in the C_4 species maize following a short dark period (5, 13). The selected illumination times for leaf exposures correspond to different phases of induction during which aspartate is the predominant initial product after 5 min of illumination, with malate formation being much more prevalent during the first few min of induction and again as the leaf metabolism approaches steady state conditions (Fig. 1).

Following a 10 s pulse, less of the ^{14}C-label was recovered in the C_4 acids with increasing time of illumination (96–79%, Fig. 2). This indicates that as the photosynthetic rates increased, a more rapid transfer of label to the RPP pathway was observed even during the pulse, as expected. However, during the last 60 s of the 2 min chase period, the total label in the C_4 acids was higher in leaves illuminated 20 min relative to those illuminated 5 min. This anomaly was not due to different rates of turnover of label from the C-4 position of the C_4 acids (Fig. 3). After either 5 or 20 min of illumination, turnover of the C-4 position (malate plus aspartate) had a calculated half-time of about 16 to 19 s (*versus* about 65 s after 30 s illumination). These turnover rates are slightly faster than estimated rates following pulse/chase experiments during steady state conditions in *Gomphrena* (8), but are slightly slower than in *Chloris* (3).

The rate of decrease in label in the C-4 position of malate plus aspartate and appearance of label in PGA and sugar phosphates can be taken as some measure of transfer of carbon to the RPP pathway. However, there is uncertainty in analyzing aspartate and malate individually as donors in *F. trinervia* since aspartate is likely metabolized to malate in the bundle sheath chloroplast prior to decarboxylation (6) as proposed in *Gomphrena* (8). This would produce a mixing of the C_4 acid pools in the bundle sheath, and would cause an underestimation of the rate of transfer of carbon from malate to the RPP pathway. This may account for the initial lag in loss of label from the C-4 position of malate, which occurs during the time of the major portion of carbon transfer from the C-4 position of aspartate (the first 10–20 s of the chase periods, Fig. 3).

The turnover rate of [4-^{14}C]aspartate was faster after 5 min illumination ($t_{1/2}$ = 8 s) compared to after 20 min illumination ($t_{1/2}$ = 30 s, Fig. 3). The faster turnover rate coincides with a large increase in carbon flux through aspartate (Fig. 1) and a transient doubling in total leaf aspartate content between 5 and 7.5 min after illumination (7). These responses most likely reflect a transient increase in the size of the photosynthetically active aspartate pool. An increased aspartate concentration gradient and presumed increased intercellular transport rate could account for the increased carbon flux through aspartate plus the faster turnover rate observed after 5 min illumination.

Analyses of the specific labeling of the C-4 position relative to

the total label in each C_4 acid indicate that during the chase period there was a continual decrease in the specific labeling of the C-4 position of each C_4 acid (Fig. 4). This decrease was considerably more rapid following 20 min of illumination, relative to after 30 s or 5 min of illumination. That is, a greater rate of appearance of label in carbons 1→3 of the C_4 acids (relative to loss of label from the C-4 carboxyl) was observed during the chase period in leaves which had received 20 min of illumination. Furthermore, this increased relative labeling rate of carbons 1→3 occurred even though the turnover rates of the C-4 position of each C_4 acid were similar (malate), or faster (aspartate), after 5 min illumination (*versus* 20 min, Fig. 3). It has been suggested that label in the C-4 position of malate can be randomized to the C-1 position through interconversion via fumarase (3). However, this is unlikely to account for the facts that both aspartate and malate showed similar patterns of percentage label in carbons 1→3 (following pulse/chase after a given illumination time), and that there was greater labeling in carbons 1→3 after 20 min of illumination. Rather, it appears that as photosynthesis approaches steady state conditions there is a considerable carbon flux from PGA of the RPP pathway to PEP of the C_4 cycle. This process could account for the above noted increase in the total percent label in the C_4 acids observed during the 60 to 120 s chase period in leaves which had received 20 min illumination, relative to 5 min illumination (Fig. 2). This might occur either through a net conversion of carbon from PGA to PEP or by an exchange reaction involving labeled PGA and unlabeled PEP (via action of PGA mutase and enolase).

During the chase period in leaves which had been illuminated for 5 min, there was a transient, unexplainable increase in the appearance of label in PGA, reaching almost 18% of the total labeled products (Fig. 5). This surge in labeling in PGA occurs as photosynthesis is approaching a maximum rate. Whole leaf PGA levels in *F. trinervia* show only a small, gradual increase throughout the induction period (7). As metabolic conditions change during induction, carbon flux through PGA likely will have different fates. The present observation may reflect some sort of transition period in the plant's utilization of PGA. Maximum labeling in sugar phosphates during the chase period in all cases was between 15 and 20% of the total labeled products.

Labeling of alanine was slower, but showed maximum percentage incorporations during the chase period following the shorter illumination times (Fig. 5). With the 30 s illumination treatment, the amount of label in alanine reached 25% of the total labeled products observed during the chase period. This labeling of alanine, which becomes more rapid with longer periods of illumination, may represent flux of carbon from labeled PGA into three-carbon metabolites of the C_4 cycle. During induction in *F. trinervia*, there is an initial transient increase in total leaf alanine content after 1 min of illumination, followed by a large, gradual decrease until after 20 min of illumination (7). A smaller, active alanine pool which is rapidly turning over during the latter part of induction may account for the lower percentage of labeled alanine appearing during the chase period in leaves receiving 5 and 20 min of illumination.

During later times of induction, glycolate was an initial photosynthetic product, containing 9% of the total label after the pulse following 20 min of illumination (Fig. 5). This occurred even though PGA labeling at this time was still very low (about 1%). Thus, the metabolic events leading to glycolate labeling after just the 10 s pulse of $^{14}CO_2$ are not clear, since labeling of the RPP intermediates (*e.g.* RuBP) was minimal at this time. Zelitch (14) reported that labeled glycolate is formed in maize leaf discs following exposure to [3-^{14}C]pyruvate, suggesting a possible alternate pathway to glycolate formation in some C_4 species. Again, however, the required sequence of events to produce a labeled putative precursor in such a short time are not clear. Glycolate also accumulated more label during the chase periods (maximally 3, 13, and 17% after 30 s, 5 min, and 20 min of illumination, respectively) with the peak accumulations observed at shorter chase times following longer periods of illumination. The observed maxima of glycolate labeling preceded the formation of [^{14}C]serine/glycine during the chase periods, suggesting the glycolate may be metabolized through the glycolate pathway.

In summary, the results of this study indicate that during photosynthetic induction in *F. trinervia*, malate and aspartate are both metabolized in the bundle sheath cells via the C_4 cycle. However, the turnover rate of the C-4 position of the C_4 acids was much slower after 30 s illumination relative to later times. The turnover rate of the C-4 position of malate plus aspartate was similar following 5 or 20 min illumination, but the appearance of label in carbons 1→3 of malate or aspartate during the chase period was much faster after 20 min illumination. The implied source of label to carbons 1→3 of the C_4 acids is an apparent carbon flux from PGA of the RPP pathway to PEP of the C_4 cycle. This may be reflecting a later event in the development of intercellular gradients of three carbon metabolites during induction in this C_4 plant.

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