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Control of Diurnal Variations in Photosynthetic Products

I. CARBON METABOLISM

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ABSTRACT

It has been demonstrated previously that the synthesis of amino acids from photosynthetically fixed carbon in leaves of *Capsicum annuum* L. cv. California Wonder occurs in the middle of the photoperiod. This paper reports experiments which identify control points regulating the carbon flow in these leaves.

Estimations have been made of the levels of intermediates between 3-phosphoglycerate and pyruvate and between 3-phosphoglycerate and fructose 6-phosphate in leaves at different times in the photoperiod. Application of the Chance crossover analysis indicates that during periods of amino acid synthesis, pyruvate kinase is activated, possibly by ammonium ions. Fructose diphosphate aldolase could possibly be an additional control point, showing activation when amino acid synthesis has ceased. There was no indication of diurnal periodicity in the activity of fructose diphosphate aldolase.

A previous study (21) indicated that a major factor in diurnal periodicity in the nature of photosynthetic products was a concomitant periodicity in the availability of reduced nitrogen in the leaf. The investigation has been continued by measuring the levels of carbon intermediates in the leaves of *Capsicum annuum*.[1]

The crossover analysis (7) was originally developed for a study of the mitochondrial electron transport pathway but has been used successfully in analysis of control points in glycolytic carbon metabolism in yeast cells (10), in aging carrot root tissue (1), and in senescing leaves (16). The method has been used here to identify control points in the steps between 3PGA[2] and pyruvate and between 3PGA and F6P, in photosynthesizing leaves during different phases of amino acid synthesis in the first 7 hr of the photoperiod.

MATERIALS AND METHODS

Plant Material. Seeds of *Capsicum annuum* L. cv. California Wonder were obtained from Arthur Yates & Co., Sydney. They were sown in a seed tray and after approximately 19 days were transplanted to pots containing a soil-sand mixture. The plants were fed daily with a nutrient solution (N2P2 of ref. 6) having 6 meq/l of NO_3^-.

The plants were raised in a glasshouse under the prevailing photoperiod. The glasshouse conditions and the method of selecting experimental material were outlined previously (20, 21). On the experimental day, plants were kept in a controlled environment facility with a constant illumination giving 107 $w \cdot m^{-2}$ (400–700 nm) at the leaf surface.

Enzyme Sources. The following ammonium sulphate suspensions were obtained from the Sigma Chemical Co. and Calbiochem Pty. Ltd.: pyruvate kinase; (EC 2.7.1.40) lactic dehydrogenase; (EC 1.1.1.2) enolase; (EC 4.2.1.11) phosphoglycerate mutase; (EC 2.7.5.3) α-glycerophosphate dehydrogenase; (EC 1.1.1.8) triosephosphate isomerase; (EC 5.3.1.1) aldolase; (EC 4.1.2.7) F6P kinase; (EC 2.7.1.11). All were preparations from rabbit muscle except enolase which was from yeast.

Aldolase Assay. Aldolase (EC 4.1.2.7) was assayed in leaf homogenates prepared in 50 mM tris-H_2SO_4 buffer pH 8.0 containing 10 mM $MgCl_2$ and 1 mM dithiothreitol. After filtration through Miracloth and centrifugation, the supernatant was used for spectrophotometric estimation of aldolase activity. The reaction mixture consisted of 150 μmoles of tris-chloride, pH 7.5 0.24 μmole of NADH; 32 IU of α-glycerophosphate dehydrogenase; 60 IU of triose-P isomerase and supernatant to make a total volume of 1.21 ml. The reaction was started by adding 1 μmole of FDP. Controls minus FDP or α-glycerophosphate dehydrogenase were run in parallel.

Extraction of Intermediates. The extraction process of Macnicol (15) was used. Two leaves for each sample were ground at 4 C with 4 ml of 5% (w/v) trichloroacetic acid containing 0.05% (w/v) 8-hydroxyquinoline. After centrifugation the pellet was re-extracted. The combined extracts were washed 6 times with 4.5 ml of cold diethyl ether (−20 C). The combined ether washes were then extracted with three lots of 8 ml of 25 mM triethanolamine chloride, pH 7.5, and the aqueous phases were added to the original ether-extracted aqueous phase. Residual ether was removed from the aqueous solution of intermediates by bubbling with N_2, this was followed by passage through columns of PVP (Polyclar AT) and DEAE-cellulose. The DEAE-cellulose eluate containing 250 mM triethanolamine chloride buffer, pH 7.5, was used for the assay of intermediates. The validity of the extraction procedure was tested by determining the recovery of authentic compounds. The following recoveries were obtained with known solutions: PYR 99%; PEP 83%; 2PGA 100%; 3PGA 68%; GAP 101%; DHAP 100%; FDP 99%; F6P 91%.

Amino acids were not retained by the DEAE-cellulose column and eluted with the loading solution front. Total amino

[1] To bring the nomenclature in line with current usage the specific *annuum* is now used, instead of *frutescens* used in previous reports.

[2] Abbreviations: 3PGA: 3-phosphoglycerate; PYR: pyruvate; PEP: phosphoenolpyruvate; 2PGA: 2-phosphoglycerate; DiPGA: 1,3-diphosphoglycerate; GAP: glyceraldehyde 3-phosphate; DHAP: dihydroxyacetone phosphate; FDP: fructose 1,6-diphosphate; F6P: fructose 6-phosphate; NO3R: nitrate reductase.

acids were determined in an aliquot using the method of Yemm and Cocking (27).

Assay of Intermediates. Spectrophotometric enzymic assays of intermediates using coupling to a dehydrogenase (4) were made in two sequences.

Pyruvate was assayed using a reaction mixture containing 60 μmoles of tris-chloride, pH 7.6; 60 μmoles of KCl; 0.1 μmole of $MnSO_4$; 0.2 μmole of ADP; 0.2 μmole of 2,3-diphosphoglycerate; 0.2 μmole of NADH in 20 mM EDTA and sample. The reaction was started by adding 86 IU of lactic dehydrogenase and recording changes in absorbance at 340 nm. The total volume was 1.13 ml. Then PEP, 2PGA, and 3PGA were determined sequentially by adding 37 IU of pyruvate kinase, 13 IU of enolase, and 5 IU of phosphoglycerate mutase, respectively.

DHAP was assayed in the presence of 60 μmoles of tris-chloride, pH 7.6; 0.2 μmole of NADH in 20 mM EDTA and sample. The reaction in a total volume of 1.04 ml, was started by the addition of 20 IU of α-glycerophosphate dehydrogenase. After completion, GAP was assayed by the addition of 60 IU of triose-P isomerase, FDP by the addition of 2 IU of aldolase. When F6P was measured after the FDP assay was complete the cuvette contained 1.7 μmoles of ATP and 5 μmoles of $MgCl_2$ in addition to the above, and the reaction was started by the addition of 13 IU of F6P kinase.

All reactions were complete within 5 min. Variable sample volumes were assayed and the results are presented in Table I.

Crossover Analyses. In the crossover analysis (7), crossovers are meaningful in two instances; when the carbon flux through a series is increasing, a forward crossover (depletion of intermediate A and accumulation of intermediate B) indicates an activation of the enzyme. When the flux is decreasing then a backward crossover (accumulation of A and depletion of B) indicates a deactivation of the enzyme. Changes in concentrations of intermediates C from samples taken at times 1 and 2 (t_1, t_2) were calculated by $100 \times (C_{t2} - C_{t1})/0.5\ (C_{t2} + C_{t1})$.

RESULTS

The Chance crossover analysis demands an increasing or decreasing flux through the sequence being studied. From the evidence reported previously (21), it seemed reasonable to accept that during the period of accumulation of amino acids, in the first 5 hr of the photoperiod, carbon flow from 3PGA to PYR is higher than during the 6th to 10th hr when amino acid levels declined. The flow of carbon from 3PGA to the sugars would present the reverse situation. In the experiment reported in Figure 1, leaves were sampled at 2.5, 4, 5.5 and 7 hr after the photoperiod started. It was anticipated that the first three samples would be in the amino acid accumulation phase and the fourth would be in the phase of predominant carbon flow to sugars. By crossover analysis between different pairs of samples, steps controlling the changing carbon flow could be identified. Fortuitously, the 4-hr sample was taken during the pause in amino acid production. This feature is illustrated in Figure 3 of reference 22. Here an experiment done in parallel to that in Figure 1 demonstrates that at 4 hr after the start of the photoperiod carbon flow into amino acids has temporarily ceased. This pattern has been observed previously (*e.g.* Fig. 1a, ref. 21).

The second sample in the crossover analysis taken at 4 hr provides information on the mechanics of the pause. A comparison of the 2.5- and 4-hr samples (Fig. 1a) shows a back-

Table 1. *Levels of Intermediates Recovered from Leaves at Different Times in the Photoperiod*

Metabolite	Time of Sampling			
	2.5 hr	4 hr	5.5 hr	7 hr
	nmoles cm⁻² ± SE of means			
PYR	4.12 ± 0.41	3.25 ± 0.38	8.43 ± 1.69	2.33 ± 0.52
PEP	1.10 ± 0.61	8.29 ± 1.64	2.47 ± 0.37	2.70 ± 0.07
2PGA	1.31 ± 0.56	2.14 ± 0.24	1.99 ± 0.18	2.54 ± 0.14
3PGA	2.85 ± 0.06	2.10 ± 0.11	3.06 ± 0.12	7.03 ± 1.20
GAP	5.84 ± 0.36	6.47 ± 0.38	6.92 ± 0.61	4.53 ± 0.25
DHAP	3.87 ± 0.45	4.32 ± 0.44	5.10 ± 1.28	2.31 ± 0.12
FDP	1.52 ± 0.40	3.17 ± 0.71	2.13 ± 0.52	3.32 ± 0.59
F6P[1]	0.78	5.50	1.99	3.41
Fresh wt (mg cm⁻²)	23.11	24.33	26.16	21.71
Amino acids, relative units (cm⁻²)	1.39	2.51	3.34	2.12

[1] Only single estimations made of F6P

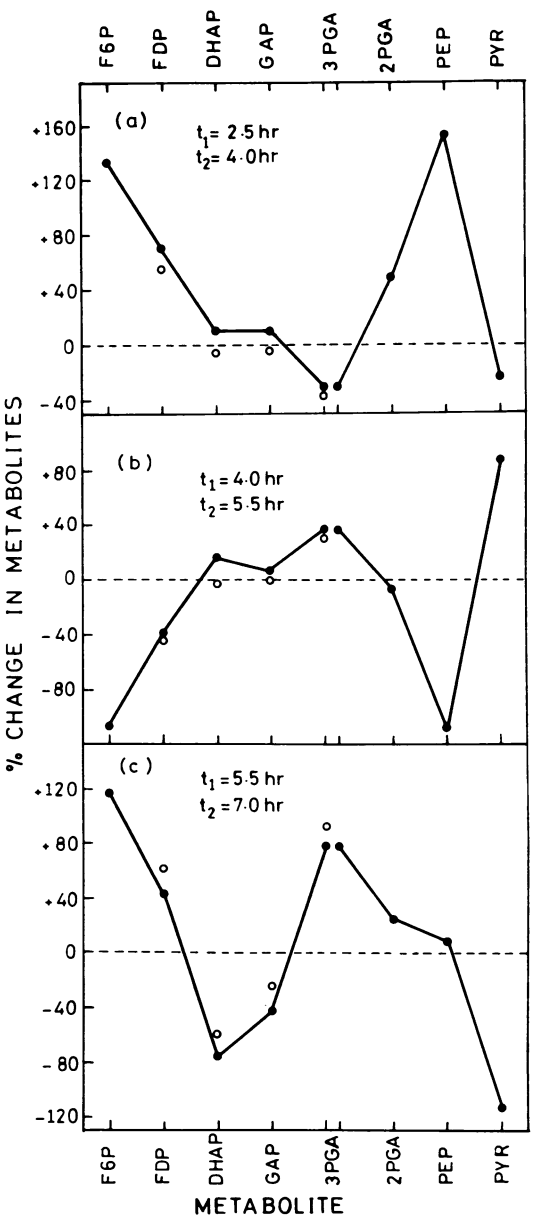

FIG. 1. Crossover analysis for intermediates from leaves. Photoperiod 05.13 to 18.35 hr and temperature 24.5 ± 0.5 C. Leaf no. 3 with leaf plastochron index between 5.6 and 6.5 and area close to 17.5 cm². Calculated from nmole/unit laminar area (●); calculated from nmole g^{-1} fresh weight for comparison (○).

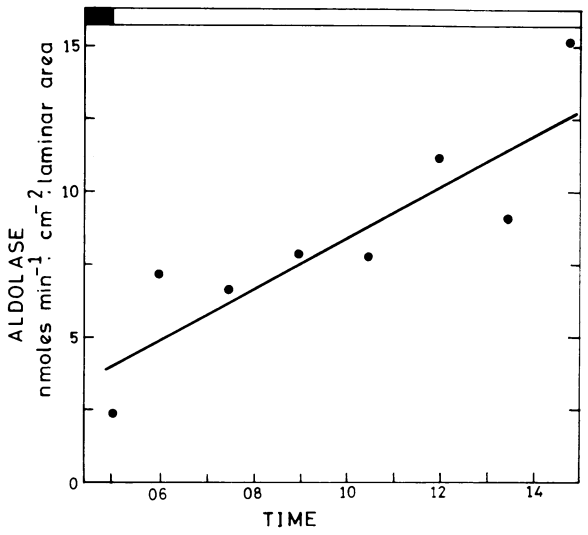

FIG. 2. Fructose diphosphate aldolase activity. Photoperiod 04.57 to 19.20 hr and temperature 26 ± 1C. Leaf no. 4 with leaf plastochron index between 5 and 6 and area about 22 cm^2.

ward crossover between PEP and PYR. Since the flow from 3PGA is less at 4 hr than at 2.5 hr, this crossover is meaningful in the crossover theory, indicating a deactivation of pyruvate kinase. Activation of pyruvate kinase is seen in Figure 1b when flow to the tricarboxylic acid cycle has recommenced and this is followed by a further deactivation (Fig. 1c) when amino acid accumulation has ceased at 7 hr. In another experiment (not shown) a clear activation of pyruvate kinase was seen between 0.5 and 2.5 hr of the photoperiod.

The other feature of this experiment that is worth noting is the level of FDP. When PEP increases (*i.e.* during deactivation of pyruvate kinase) FDP increases, when PEP decreases (*i.e.* during activation of pyruvate kinase) FDP decreases. The change in the triose phosphates is usually small and not significantly different from zero change (See Table I). Thus it is not clear whether the change in FDP content reflects an activation and inactivation of aldolase except perhaps between 5.5 and 7 hr when triose phosphate changes may be considered significant. The low GAP:DHAP ratios may be a result of pool mixing during extraction, for the expected values would be higher.

The activity of aldolase has been shown to exhibit diurnal periodicity in tapioca leaves (25) and the leaves of the succulent, *Nopelea dejecta* (18). However, in *Capsicum* there was a steady increase in aldolase activity throughout the photoperiod (Fig. 2) but no indication of a periodicity that would explain the levels of FDP seen in Figure 1. Metabolite feedback control of aldolase activity is possible, but no information for the enzyme from this species is available. There was no indication from the crossover analysis of an activation of glyceraldehyde-3-P dehydrogenase by accumulated amino acids (24).

The activation of pyruvate kinase during periods of amino acid accumulation is consistent with an activation by NH_4^+ (17). This again emphasizes the important role that the availability of reduced nitrogen plays in the control of photosynthetic products in this species.

DISCUSSION

Cautious use must be made of the crossover analysis in carbon metabolism, for the abundance of branched pathways and compartmentation of separate pools of intermediates could lead to confusing results. Nevertheless, the consistent indication of pyruvate kinase as a control point in carbon metabolism reported here, together with the finding that the labeling of citrate was high when the labeling of amino acids was high (21), suggests an activation of this enzyme as a prime control point in carbon flow in photosynthesizing *Capsicum* leaves. Studies of the regulation of photosynthetic carbon metabolism in *Chlorella* showed that the addition of NH_4Cl to the culture caused immediate changes in the levels of PEP and PYR (12). Together with the known activation of seed pyruvic kinase by NH_4^+ (17), this points to NH_4^+ activation of the enzyme in photosynthetic tissues in higher plants.

Interpretation of the analyses of the series of intermediates 3PGA to F6P must be more cautious, for these are intermediates both of the cyclic reductive pentose phosphate pathway and the glycolytic pathway. To what extent intermediates destined for synthesis of the CO_2 acceptor or carbohydrate synthesis are compartmentalized either inside or outside the chloroplast is unknown.

The changes in FDP levels, and the parallel changes in F6P, cannot be satisfactorily explained by the known regulators of plant F6P kinase. These include inhibition by ATP, citrate (8), and PEP (13) and stimulation by phosphate (8). Likewise, the inhibition of FDPase by nitrite (11) does not provide an explanation. The crossover analysis gives no indication of a controlled reaction between FDP and F6P. In addition plant pyruvate kinase, unlike the enzyme from animal sources, is not activated by FDP (9) so that the high FDP levels occurring when pyruvic kinase is deactivated is not an anomaly in this tissue.

If carbon destined for carbohydrate synthesis is removed from the reductive pentose phosphate cycle pools at an early intermediate (*e.g.* DHAP), then control at the aldolase step could be anticipated. Stocking and Larson (23) have suggested that the NADH required by nitrate reductase is generated outside the chloroplast by the oxidation of GAP by glyceraldehyde-3-P dehydrogenase. As triose-P is chloroplast derived, this provides a shuttle mechanism across the chloroplast membrane for reducing potential. However, not only will this translocated triose-P provide extra-chloroplastic reducing potential but also carbon skeletons for metabolism to PEP. If the alternative fate of the extra-chloroplastic triose-P is conversion to sucrose then aldolase is seen to be a potential control point. A modification of the Stocking and Larson scheme (23) is presented in Figure 3.

Klepper *et al.* (14) came to a similar conclusion in their studies of the shuttle for the reducing potential for NO3R. Their scheme shows GAP, rather than DHAP, as the shuttle compound and provides for a chloroplastic and cytoplasmic

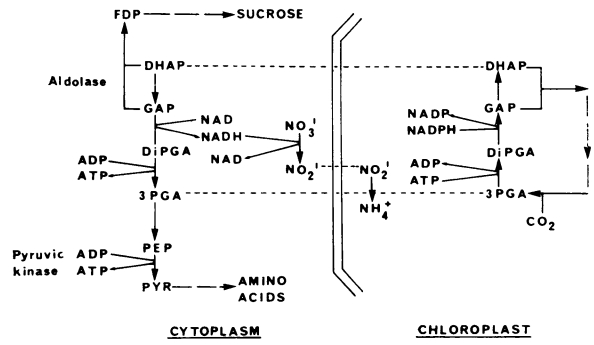

FIG. 3. Scheme of possible localization of controlled steps in synthesis of amino acids and sucrose from CO_2. The final steps in both syntheses may occur in other compartments.

pool of GAP, but not of DHAP; a concept that may explain the low GAP:DHAP ratios in *Capsicum* leaves. In this case, FDP may be envisaged as the transport compound leading to extra-chloroplastic carbohydrate synthesis (5). An increase in the extra-chloroplastic pools of FDP and F6P could occur when amino acid synthesis is low and carbohydrate synthesis is high. Such a scheme would have no role for an extra-chloroplastic aldolase during photosynthesis and may explain the crossover patterns as well as the concept of aldolase as a control point, except for the decrease in GAP and DHAP between 5.5 and 7 hr.

Anderson and Advani (2) have demonstrated from pea leaves, by isoelectric focusing, that chloroplastic and cytosolic forms of FDP aldolase, triose phosphate isomerase, and 3PGA kinase are different. However, nothing is known of the regulation of type 1 aldolase found in photosynthetic tissues (26) beyond that it does not require a divalent cation nor high K^+ concentrations for activity and is unaffected by chelating agents but inhibited by mercurials. These properties make aldolase control during amino acid accumulation difficult to explain. Aldolase from some animal sources is inhibited by ATP (3, 19). A close association of aldolase and pyruvate kinase could allow the ATP produced by the latter to exert some control on the activity of aldolase involved in the carbohydrate synthetic pathway. However, preliminary experiments with crude preparations of aldolase from *Capsicum* have not demonstrated any inhibition by ATP levels up to 15 mM. Further investigation of aldolase in this tissue is desirable before a choice may be made between the two schemes discussed above.

The periodicity of nitrate reductase activity in the leaves of *Capsicum annuum* is reported in the accompanying paper (22), and the supply of NH_4^+ seems to be the major control point in the periodicity of photosynthetic products. It is probably NH_4^+ that activates pyruvate kinase allowing an increased synthesis of the carbon skeletons for the amino acids. In addition, the results reported here suggest that aldolase may also be a regulated enzyme limiting the flow of carbon to carbohydrates when amino acid production is high.

Note Added in Proof. There is information of modulators of pea leaf aldolase activity (Anderson, L. E. and I. Pacold. 1972. Plant Physiol. 49: 393–397). The results reported there suggest that the role of citrate levels in the control of aldolase activity in *Capsicum* leaves would repay investigation.

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