Diurnal Variations in Photosynthetic Products and Nitrogen Metabolism in Expanding Leaves

BARRIE T. STEER
Division of Irrigation Research, Commonwealth Scientific and Industrial Research Organization, Griffith, N.S.W., Australia

ABSTRACT
Expanding leaves of Capsicum frutescens L. cv. California Wonder, Cucumis melo L. cv. Hales Best, and Citrus sinensis L. Osbeck cv. Washington Navel showed a marked diurnal periodicity in the incorporation of 14C from photosynthetically fixed 14CO2 into amino acids. Incorporation was virtually nil at the beginning of the photoperiod, reached a maximum in the 6th to 7th hour and decreased during the latter part of the photoperiod. In Capsicum frutescens this was apparently a reflection of the availability of reduced nitrogen controlled by the activity of nitrate reductase in the leaves. This also controlled the periodicity of the incorporation of 14C into fraction I protein. Possible control mechanisms and the relation of nitrogen metabolism to the periodicity of leaf expansion growth are discussed.

Despite the extensive literature on carbon metabolism in photosynthesis in higher plants, there is little information on variations in the flow of carbon to photosynthetic products with respect to the time of day. Before the influence of environmental factors on photosynthetic carbon metabolism can be studied, it seems necessary to establish the basic patterns of diurnal variations in metabolism. Filippova (8) has pointed out that labeling of products in leaves of a range of plants during a 10-min presentation period of 14CO2 depends very much on the type of carbon metabolism in the different species, but that in general the flow of carbon to noncarbohydrate compounds is greater in the morning than in the afternoon. This report presents similar findings and suggests some controlling factors, including nitrate reductase activity, involved in diurnal photosynthetic carbon flow in leaves of Capsicum frutescens.

METHODS AND MATERIALS
Plant Material. Seeds of Capsicum frutescens L. cv. California Wonder (green pepper) and Cucumis melo L. cv. Hales Best (melon) (Arthur Yates and Co. Pty., Sydney) were sown in soil or a sand-pea mixture in pots in a greenhouse. Details of different nutritional practices are reported in the “Results” section. The minimum temperature was never below 21°C, but the maximum varied with the ambient. Citrus sinensis L. Osbeck cv. Washington Navel were greenhouse-grown cuttings about 2 years old and raised according to Cary (7).

The experimental material was in all cases intact expanding leaves, and in any one experiment the leaves used were of the same insertion number. Comparable leaves of Capsicum frutescens and Cucumis melo were selected on the basis of laminar area and leaf plastochron index using an arbitrary leaf length value of 10 mm (17, 18) and 20 mm for the respective species. In the experiments reported, leaf 2 of Capsicum frutescens has an LPI of about 5.0 and an area of 10 cm², and leaf 3 an LPI of 8.0 and an area of 10 cm². The values for Cucumis melo were LPI of 1.40 and area 38 cm². LPI values were not estimated for theflush of Citrus sinensis, but half-expanded leaves were used with a leaf area of close to 17 cm².

14CO2 Presentation and Isolation of Labeled Compounds. The experimental plants were moved from the greenhouse to a controlled temperature and light environment on the afternoon preceding the experimental day. The day length of the experimental day was the same as the current natural day, but the incident light intensity and temperature (25 ± 1.5°C) were constant. In early experiments the lighting was provided by fluorescent lamps, but later experiments used a combination of metal arc and tungsten lamps. Light intensity during each experiment was measured with an ISCO spectroradiometer and is quoted in the “Results” section for the waveband 400 to 700 nm.

Trace amounts of 14CO2 were used to supplement air, and, assuming the CO2 concentration to be 300 μl/liter, the level of radioactivity provided was 20 μCi in 10 μmoles of CO2. In an experiment where the CO2 concentration was doubled to an assumed 600 μl/liter the pattern of variation in photosynthetic products was no different from that in normal CO2 levels.

Leaves were presented with 14CO2 in an acrylic leaf chamber in a gas-circulating system comprised of a gas generator, water scrubber, pump, and gas switches. The system could be reversibly switched from open to closed circuit, and 14CO2 was released from NaOH·14CO2 by acid injection when the system was in the closed circuit mode. Exposure to 14CO2 was for 5 min in all cases. Preliminary experiments showed that 14C incorporation occurred in a linear manner into all major soluble components during that period but that none attained a steady state with respect to labeling. At the end of 5 min the circulator was switched to open circuit, remaining 14CO2 was absorbed in NaOH, and the leaf was rapidly removed from the chamber and placed in boiling 80% (v/v) ethanol.

After 5 min of boiling the leaf was left in ethanol at room temperature overnight. This was followed by a further extraction with boiling 80% ethanol and two extractions with boiling water. The ethanol and water extracts were combined and dried on a rotary evaporator, and chloroform-soluble components were removed by washing the solids with chloroform. The residue was dissolved in water and subjected to column ion exchange chromatography using Dowex 50W X8 (H+) and

1 Abbreviation: LPI: leaf plastochron index.
were labeled in Washington Navel and Capsicum expressed as a percentage of the total recovered. Only those while citrate was labeled only in the latter.

The residue from the ethanol-water extraction was extracted with 0.5 N NaOH for 24 hr at room temperature. This fraction contained proteins, and from Sephadex G200 column chromatography monitored at 280 nm there was no evidence of the presence of \( ^{14} \text{C} \)-labeled compounds with molecular weight of less than 5000. All radioactivity was associated with compounds at 280 nm and giving a positive reaction in the Folin test for proteins. For these reasons the fraction is called the protein fraction throughout the text.

Insoluble material remaining after the NaOH extraction was incubated with 30% (w/w) perchloric acid for 24 hr at room temperature. Starch was precipitated from this acid extract with \( \text{I}_2/\text{Kl} \) in the presence of 5% (w/v) NaCl and removed by centrifugation. The starch was solubilized by hydrolysis in 2 N HCl on a boiling water bath for at least 6 hr.

The insoluble material remaining after this series of extractions was divided finely in an homogenizer, suspended in a fluor solution with silicon dioxide (Cab-o-sil, Packard Instrument Co.) to give a 4% gel and assayed for radioactivity.

Aliquots of the other fractions (basic soluble, acidic soluble, neutral soluble, chloroform-soluble, protein, starch, and perchloric acid-soluble) were assayed for \( ^{14} \text{C} \) content in a scintillation spectrometer using a toluene-dioxane fluor. Activity was corrected for background and variable quenching using an external standard facility and a computer program. Further separation of the components of fractions was accomplished by either paper chromatography or column chromatography using standard amino acid analyzer systems.

Samples of chromatography paper were cut out and counted directly in a toluene-PPO-POPOP fluor solution. Aqueous samples were counted as above.

**Nitrate Reductase Assay.** Nitrate reductase activity was assayed in leaf homogenates prepared in 100 mM phosphate buffer, pH 7.5, containing 1 mM dithiothreitol and 1 mM EDTA and filtered through muslin. Enzyme activity was assayed by the method of Hewit and Nicholas (10) with a control minus NADH and calibrated using standard nitrite solutions. Nitrate reductase activity is expressed as nanomoles of nitrite produced per hour and cm\(^2\) laminar area.

**Sap Nitrogen Measurements.** Bleeding sap was collected from samples of 10 plants having similar plastochron indices (about 10.0). At hourly intervals groups of plants were decapitated just below the level of insertion of the cotyledons, and sap was removed with a capillary pipette at intervals during the subsequent hour. Total sap volume, \( \text{NO}_3^- \) (20), \( \text{NH}_4^+ \) (4), and total N content were measured on the pooled sap.

**Expansion Rate of Leaves.** The rate of linear expansion growth was measured by attaching the leaf tip to a linear variable differential transformer in a system similar to that used by Hsiao, Acevedo, and Henderson (12). The stem was anchored at the node, and continuous recordings were made for 30 hr on a single leaf.

**RESULTS AND DISCUSSION**

In addition to the phosphorylated intermediates of the reductive pentose phosphate cycle, the \( ^{14} \text{C} \)-labeled compounds recovered after 5 min of exposure to \( ^{14} \text{CO}_2 \), expressed as percentage of total \( ^{14} \text{C} \) recovered. a: *Capsicum frutescens*. Leaf 2. Mean \( ^{14} \text{C} \) recovered from eight samples: 145 \( \pm \) 25. 10\(^3\) dpm per cm\(^2\) laminar area. b: *Cucumis melo*. Leaf 3. Mean \( ^{14} \text{C} \) recovered: 21 \( \pm \) 2. 10\(^3\) dpm. c: *Citrus sinensis*. Fourth leaf of flush bearing seven leaves. Mean \( ^{14} \text{C} \) recovered: 49 \( \pm \) 13. 10\(^3\) dpm. o: Total amino acids; \( \bigtriangleup \): sucrose; \( \Delta \): fructose; \( \bigtriangledown \): malate. Natural and experimental day length marked by arrows. Light intensity at leaf surface 100 W m\(^{-2}\) (400–700 nm).

The most striking change in photosynthetic carbon flow involves nitrogenous products, and the known diurnal periodicity of nitrate reduction systems (2) suggested that the availability of reduced nitrogen might be a controlling factor. For this reason further experiments were conducted where the levels of nitrogen in bleeding sap and leaf nitrate reductase activity were measured in addition to \( ^{14} \text{C} \)-labeling patterns in *Capsicum frutescens*.

A population of plants that had been raised in a peat-sand mixture and irrigated with a high nitrate (6 meq/liter) culture solution was used. Data on sap nitrogen levels and leaf nitrate

---

**Fig. 1.** Diurnal variations in the \( ^{14} \text{C} \) content of metabolites after 5 min of exposure to \( ^{14} \text{CO}_2 \), expressed as percentage of total \( ^{14} \text{C} \) recovered. a: *Capsicum frutescens*. Leaf 2. Mean \( ^{14} \text{C} \) recovered from eight samples: 145 \( \pm \) 25. 10\(^3\) dpm per cm\(^2\) laminar area. b: *Cucumis melo*. Leaf 3. Mean \( ^{14} \text{C} \) recovered: 21 \( \pm \) 2. 10\(^3\) dpm. c: *Citrus sinensis*. Fourth leaf of flush bearing seven leaves. Mean \( ^{14} \text{C} \) recovered: 49 \( \pm \) 13. 10\(^3\) dpm. o: Total amino acids; \( \bigtriangleup \): sucrose; \( \Delta \): fructose; \( \bigtriangledown \): malate. Natural and experimental day length marked by arrows. Light intensity at leaf surface 100 W m\(^{-2}\) (400–700 nm).
reductase activities were collected during 1 day and \(^{14}C\) distribution the following day. The results are plotted in Figure 2. Most nitrogen moves up the stem of *Capsicum frutescens* as nitrate, and thus the plant is dependent upon leaf systems for reduction to \(\text{NH}_4^+\). \(\text{NO}_3^-\) and \(\text{NH}_4^+\) account for about 80% of the total N in the sap at any time of day. Translocation of nitrate is high from the beginning of the photoperiod and then falls steadily after the 4th hr to reach a low level by the end of the photoperiod. Extracted nitrate reductase activity shows a marked diurnal variation and reaches maximal activity during the 4th hr followed by a rapid decline. This pattern is mimicked not only by the \(^{14}C\) in amino acids but also by that in fraction I protein, which accounts for most of the label in the protein fraction. Thus, the different amounts of \(^{14}C\) in the free amino acids are not due to different efflux rates to the proteins, for labeling of the protein fraction is only high when labeling of free amino acids is high. The periodicity of fraction I protein synthesis appears to be controlled by the availability of reduced nitrogen. The same pattern of \(^{14}C\) incorporation into the amino acids was obtained from plants illuminated at a low light intensity (22 \(\text{w m}^{-2}\)).

In Figure 2b nitrate reductase shows an increase in activity at the end of the photoperiod when sap nitrate has fallen to low levels. This increase was seen in other experiments both for nitrate reductase activity and sometimes in the \(^{14}C\) content of amino acids (e.g., Fig. 1a). In addition, the fall in nitrate reductase activity in the middle of the photoperiod occurs at a time when stem nitrate translocation remains high. Thus, it appears that the amount of nitrate moving up the stem is not the only determining factor in the production of reduced nitrogen in the leaf. While there is probably a control point at the diversion of nitrate from the stem to the leaf lamina (16), it is possible for an additional control point to be in the laminar cells themselves. There is evidence that amino acids can act as repressors of nitrate reductase activity in *Lemna minor* (19) and in cultured tobacco cells (9), but Beevers et al. (2) could not find a similar repression in radish cotyledons and corn leaves. Diurnal variation in the size of the free amino acid pools in *Capsicum frutescens* (Fig. 2d) is consistent with a control of nitrate reductase activity by amino acids in this species. The amino acid content is highest at the time (5th and 6th hr) when nitrate reductase activity is lowest. Just before the end of the photoperiod the amino acid content falls to a low level, and any repression would be lifted allowing an increase in nitrate reductase activity. Thus the presunset increase in enzyme activity could be a response to a low amino acid content, but this suggestion will need rigorous experimental testing. The relationship between amino acid content and nitrate reductase activity shows that any regulation of enzyme activity by amino acids has a short response time suggesting a feedback control of enzyme activity rather than a repression of enzyme synthesis.

The carbon of many amino acids is derived from intermediates of the tricarboxylic acid cycle. Entry into the cycle from 3-phosphoglyceric acid involves the step catalyzed by pyruvic kinase. Pyruvic kinase from pea seed (14) is activated by the ammonium ion, and a similar regulation in a photosynthetic organism was suggested in *Chlorella pyrenoidosa* by \(^{14}\text{CO}_2\) studies (13). In *Capsicum frutescens* the diversion of \(^{14}C\) into amino acids is accompanied by diversion into citrate (Fig. 3) consistent with an activation of pyruvic kinase by \(\text{NH}_4^+\), resulting from the increased nitrate reductase activity in the middle of the photoperiod.

Figure 2d shows that at the beginning of the photoperiod.
the free amino acid content of the leaves is very low. While a detailed analysis of the amino acids will be the subject of another report, it may be stated that individual acids exhibited the same trends as the total amino acid content. The exception was arginine, the content of which remained constant throughout the photoperiod. Arginine and lysine were the only amino acids detected in the sample taken at 06:25 hr.

The specific radioactivity of the total amino acids at the beginning and end of the photoperiod reflected the low flow of 14C into the amino acids at those times of day. Toward the middle of the photoperiod the flow of 14C into the amino acids was high relative to the pool sizes, and this was reflected in the increase in specific radioactivity. However, at 11.00 hr the 14C influx had extended to the size extent as the increase in pool sizes, and the specific radioactivity was low. With the depletion of the pools and the 14C influx remaining high, the specific radioactivity rose again, to be followed by a decrease as synthesis of amino acids ceased at the end of the photoperiod.

Berner (3) has pointed out that the time of maximal size of free amino acid pools in leaves differs among species. Capsicum frutescens is like another solanaceous species, tobacco (15), in having a maximal free amino acid content in the middle of the photoperiod and unlike Pisum arvense L. (6), which has maxima of most amino acids in the middle of the dark period. Carr and Pate (6) observed that the amino acid content in P. arvense was out of phase with the protein content of the leaves and that minima may reflect periods of rapid protein synthesis. This does not seem to be the case in Capsicum frutescens grown on adequate nitrate where the maximal carbon flow into a major protein, fraction I protein, occurs at a time when the amino acid pools are largest. Unlike P. arvense, there was no indication in Capsicum frutescens of a fluctuation in total leaf protein content with the time of day.

Bünning (5) has shown that the rate of expansion growth of leaves differs with the time of day. In Capsicum frutescens the highest rate is seen at the beginning of the photoperiod (Fig. 4) with little expansion occurring at other times. Thus, the size of the amino acid pools and the flow of carbon into fraction I protein are maximal soon after leaf expansion has ceased (Fig. 2, cf. Fig. 4). At that time increased cell wall surface will be available for chloroplast growth (11, 18), and it is not surprising that a chloroplast component, fraction I protein, shows maximal carbon incorporation at that point in time. Bünning (5) observed that some species e.g., Glycine soja (Biloxi), had a maximal leaf growth rate in the middle of the dark period and a minimum during the photoperiod. The need for protein synthesis in these species would occur at a different time of day than in Capsicum frutescens and may be reflected in large pools of amino acids being present in the middle of the dark period as in P. arvense. The relationship in different species between the periodicity of leaf growth rate and amino acid and protein synthesis may repay further investigation.

The largest variation in the flow of photosynthetically fixed carbon is that incorporated into amino acids, and results suggest that the controlling factor is the availability of reduced nitrogen. While other control points (1), such as sucrose synthetase, may play a part in diverting carbon to amino acids, these have not been investigated in the present study, and only an indication that pyruvic kinase may be activated by NH4+ has been obtained.

The controlling function of leaf nitrogen status on the synthesis of amino acids in photosynthesis makes a study of nitrogen metabolism in expanding leaves of Capsicum frutescens relevant. In particular, the influence of different forms and levels of nitrogen in the culture medium on diurnal variation of amino acid synthesis is being studied.

**Acknowledgments**—I am indebted to Mr. A. H. Gunn for the amino acid analyses and to Mr. M. L. Higgins for the nitrogen determinations.

**LITERATURE CITED**

12. HIISO, T. C., E. ACACIA AND D. W. HENDERSON. 1970. Maize leaf elonga-


