Plant Physiol. (1974) 54, 762–765

Control of Diurnal Variations in Photosynthetic Products

II. NITRATE REDUCTASE ACTIVITY

Received for publication April 9, 1974 and in revised form July 2, 1974

BARRIE T. STEER
Division of Irrigation Research, Griffith, Commonwealth Scientific and Industrial Research Organization, New South Wales, Australia

ABSTRACT

Nitrate accumulates in the leaves of *Capsicum annuum* L. cv. California Wonder and the leaf content is dependent on the nitrate level supplied to the roots. There is no consistent diurnal periodicity in the leaf nitrate levels.

Nitrate reductase activity exhibits three distinct peaks in the leaves. One in the dark period, a second at the time of the prevailing dawn, and a third 6 hours after the beginning of the photoperiod. The third peak of activity can be induced by a short period of illumination (20 minutes) which is also sufficient to induce the labeling of the amino acid fraction by $^{14}CO_2$.

The availability of reduced nitrogen plays a major role in the diurnal periodicity of photosynthetic products as shown previously (7) and in the accompanying paper (8). Wallace and Pate (9) have recorded nitrate reductase activity in leaves of *Pisum arvense* over a period of 30 hr. Activity was low in the dark period and had a maximum in the early afternoon. In leaves of *Perilla frutescens* (4) a peak in NO3R[1] activity occurred 5 to 6 hr after the beginning of illumination.

Some information is available on the behavior of nitrate reductase in *Capsicum* leaves (7), but it was thought that further investigations would reveal more details of the nitrogen/carbon interactions in photosynthesis.

This paper reports some characteristics of *Capsicum* leaf NO3R which exhibits three separable peaks of activity occurring in the 24-hr cycle. The response to short periods of illumination has also been studied.

MATERIALS AND METHODS

Plant Material. Plants of *Capsicum annuum*[2] were raised in a glasshouse (8), with the exception of those used in the experiments reported in Figures 5 and 6. Here, after transplanting to pots they were kept in a controlled environment cabinet with a 10/14 hr light/dark, 27/24C cycle, humidity between 35 and 50% and light intensity 100 $w \cdot m^{-2}$ (400–700 nm). In the experiments reported in Figures 1 and 2, $CaCl_2$ and $Ca(NO_3)_2$ contents of the nutrient solutions were adjusted to give the desired NO_3^- levels, otherwise NO_3^- was supplied at 6 $meq \cdot l^{-1}$.

Leaf NO_3^- NH_4^+ Contents. Leaves were homogenized in 2 M KCl, centrifuged, and the supernatant was assayed for NO_3^- and NH_4^+ as outlined previously (7).

Nitrate Reductase Assay. The *in vitro* method of Hewit and Nicholas (2) was used, as previously reported (7).

$^{14}CO_2$ Presentation and Isolation of Labeled Compounds. This was carried out as previously reported (7). To simplify the procedure the results in Figure 5 are reported as ^{14}C as a percentage of that in the basic, acidic, and neutral soluble fractions only and not as percentage of total recovered ^{14}C. Expression of results by either method has not altered the pattern of previous results.

RESULTS

Leaf Nitrogen Content. The periodicity of the NO_3^- content of stem sap (7), with a maximum during the 3rd hr of the photoperiod, suggested that the leaf NO_3^- content may vary and thus provide variable induction of NO3R activity with the time of day. To test this, leaf NO_3^- and NH_4^+ contents were measured at 2-hr intervals over 28 hr in plants supplied with either 0.6 or 6.0 $meq \cdot l^{-1}$ NO_3^-. Figure 1 shows that the contents of both ions remained fairly constant throughout the 28 hr despite that fact that in both populations leaf amino acid content (not shown) had maxima at 1400 hr in the first photoperiod, at 2200 hr in the dark period and at 1200 hr at the end of the experiment. Leaf NO_3^- content appeared to be a function of the NO_3^- level supplied in the culture solution (Fig. 2) and did not show any consistent periodicity, *Capsicum annuum* was similar in this latter respect to *Medicago sativa* (10). This is consistent with the bulk of NO_3^- in the leaf, being in storage pools with the metabolic pools being small in comparison. This concept is supported by results reported by Martin (6) who found evidence for compartmentation of NO_3-N into metabolic and storage pools in the leaves of *Phaseolus vulgaris*.

The large storage pools of NO_3^- made any investigation of the induction of NO3R activity from the standpoint of leaf NO_3^- content difficult and other approaches were attempted.

Nitrate Reductase. Preliminary experiments showed that the increase in NO3R activity in the photoperiod was related to the time of onset of illumination. To investigate further the light effect, the amino acid content, ^{14}C labeling patterns and the activity of NO3R were measured at various times after leaves had experienced 20 min light followed by darkness. After this treatment there was no increase in amino acid

[1] Abbreviations: NO3R: nitrate reductase; LPI., leaf plastochron index.

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

content and this was probably a reflection of the lack of photosynthetically fixed carbon. However, when the labeling of the amino acid fraction was investigated it was seen that replacing the normal photoperiod by 20 min light at dawn was sufficient to induce the normal periodicity in the flow of ^{14}C to amino acids (Fig. 3).

NO3R activity was investigated to see if the light induction of labeling of the amino acid fraction was a result of the induction of NO3R activity by brief illumination. Figure 4 shows the result of 20 min light at dawn and a comparison of activity may be made with plants kept in complete darkness. It is seen that brief illumination induces an increase in activity peaking at 6 hr after the illumination. The dark control plants show no such increase in activity although both treatments show a smaller peak about 2 hr after the normal dawn. This

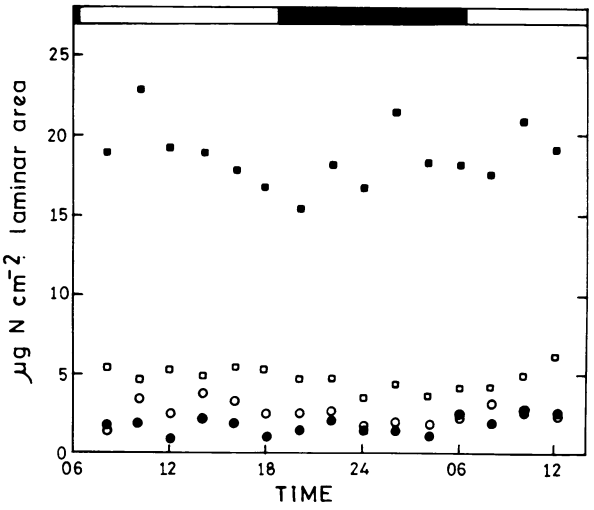

FIG. 1. Nitrate-N and ammonium-N content of leaves throughout 28 hr. From emergence to 19 days before sampling all plants were raised on 6 meq·l^{-1} NO_3^-. Thereafter some were transferred to 0.6 meq·l^{-1} NO_3^-. Light and dark periods are given by horizontal bar. Temperature was 22 ± 1C. Leaf no. 3 with L.P.I. about 5 and area 11 cm^2 for low NO_3^- series (●: NO_3-N; ○: NH_4-N) and L.P.I. about 5.7 and area 15 cm^2 for high NO_3^- series (■: NO_3-N; □: NH_4-N).

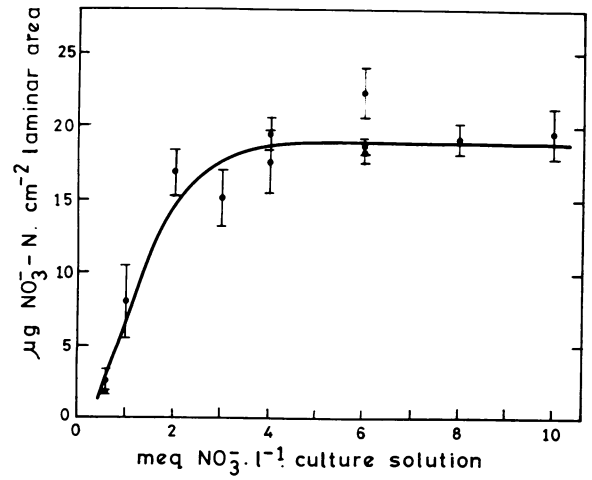

FIG. 2. Leaf NO_3-N levels as a function of the NO_3^- content of the culture solution supplied to the plants. Plants were raised in a glasshouse in a soil/sand mixture and irrigated daily with the culture solution. Leaves 3, 4, or 5 were sampled at L.P.I. 4 to 6. The bars represent the SE of the mean.

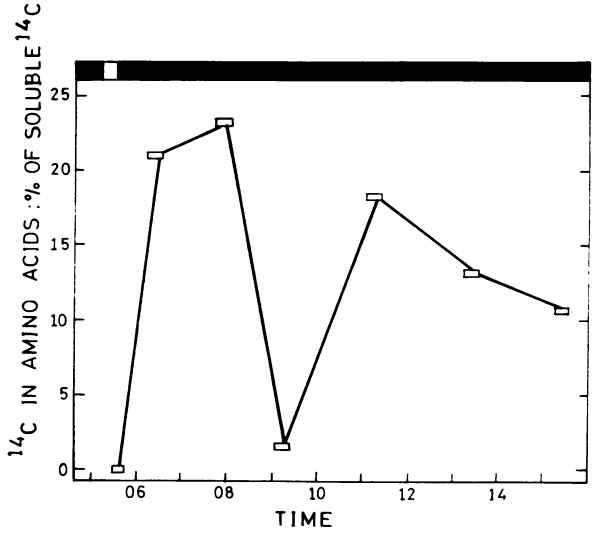

FIG. 3. Percentage of aqueous ethanol-soluble ^{14}C in amino acid fraction at intervals after 20 min inductive white light given at 05.13 to 05.33 hr. Normal photoperiod was 05.13 to 18.35 hr. At the sampling times single plants were given 15 min light for photosynthetic induction followed by 5 min exposure of leaf number 2 to $^{14}CO_2$ (20μCi) in the light. Temperature was 24.5 ± 0.5C. Leaf number 2 with L.P.I. about 8.5 and area about 16.5 cm^2.

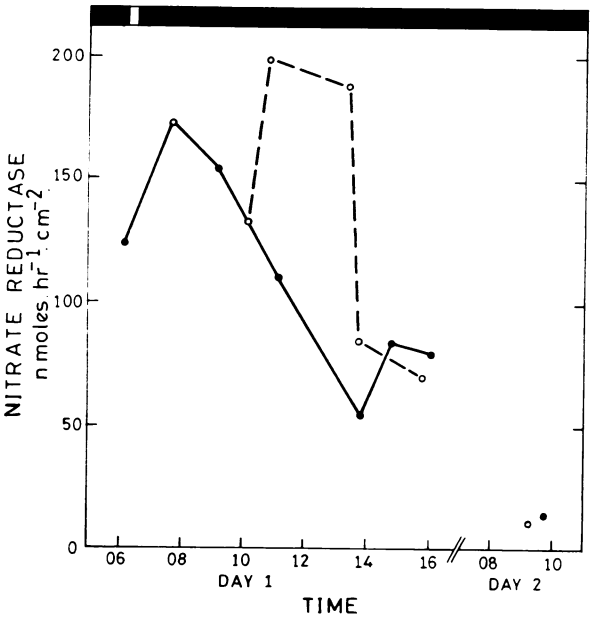

FIG. 4. Nitrate reductase activity after 20 min white light (06.13 to 06.33 hr) (○) or in continuous darkness (●). Normal photoperiod 06.13 to 20.25 hr. Leaf number 5 with L.P.I. about 5 and area about 15 cm^2. Temperature was 25 ± 1C.

activity peak is obviously not dependent upon concurrent illumination but is dependent upon illumination during the previous day, as the activities measured on day 2 are similar for both treatments and both are low.

These peaks of NO3R activity are illustrated further in Figures 5 and 6. In Figure 5, a decline in activity in the latter part of the first photoperiod is evident, and this is followed by a peak in the dark period (peak I). The beginning of this peak has sometimes been seen in the latter part of a photoperiod (Fig. 1, a and b, ref. 7). By extending the dark period (Fig. 5) the early morning peak (peak II) is clearly

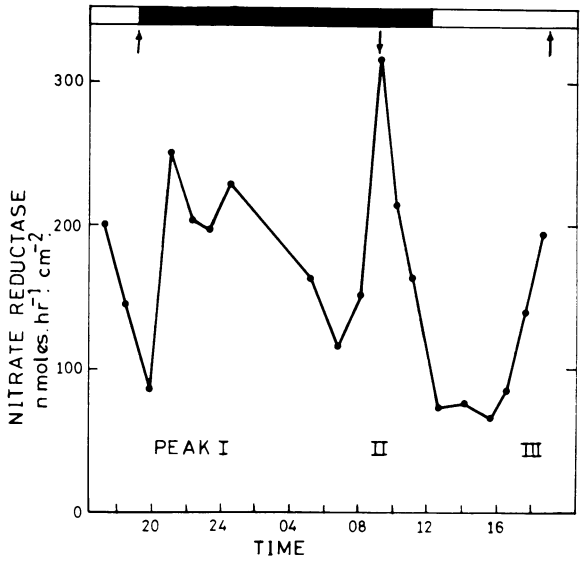

FIG. 5. Three peaks of nitrate reductase activity in leaves. Plants raised in a controlled environment cabinet with normal photoperiod (09.05 to 19.10 hr) shown by arrows. Experimental dark period was extended by 3 hr as shown by horizontal bar. Leaf number 3 with L.P.I. about 3 and area about 29 cm². Temperature was 26 ± 1C.

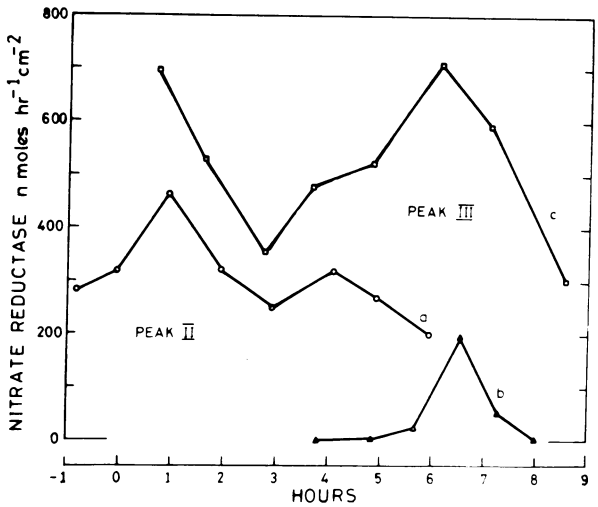

FIG. 6. Typical peaks II and III of nitrate reductase activity in leaves from plants raised in a 10-hr photoperiod. Time zero is the time of the prevailing dawn and, in b is the time of red irradiation. a: 24 hr continuous light previous to time zero. Leaf number 5 with L.P.I. about 4 and area 11 cm². Temperature was 26 ± 1C. b: 20 min red light (630–690 nm; 123 μw cm²) at time zero then darkness. Plants raised on low (0.3 meq/l) NO_3^-. Leaf number 8 with L.P.I. about 2 and area 6.5 cm². Temperature was 23 ± 1C. c: Plants exposed to normal, 10-hr photoperiod at time zero. Leaf number 6 with L.P.I. about 4.9 and area 8 cm². Temperature was 25.5C.

defined and shows maximum activity at the time of the normal dawn (0900 hours). When the photoperiod was started 3 hr later than normal (1200 hours) this induced the other NO3R activity peak (peak III), reaching a maximum about 6 hr after the beginning of illumination.

Figure 6 is a composite illustration of three separate experiments designed to show (*a*) the occurrence of peak II at the time of normal dawn in plants where the preceding dark period has been replaced by continuous light; (*b*) the induction of peak III in leaves of low nitrate plants after exposure to 20 min of red light; (*c*) for comparison, NO3R activity of leaves experiencing a normal photoperiod. Despite leaf position, age and experimental temperature differences the timing of the peaks was coincidental. This was so in plot *a* where the continuous 24 hr light produced a dampened peak II and in plot *b* where the low NO_3^- status of the plants resulted in low NO3R activity.

Thus, there are three peaks of NO3R activity, two occurring wholly or partially in the dark period and one induced by brief periods of illumination and normally evident during the middle of the photoperiod. The timing of the early morning peak (peak II) and the light-induced peak (peak III) explain the pause in ^{14}C labeling of amino acids (Fig. 3) and the deactivation of pyruvate kinase at about the 4th hr of the photoperiod (Fig. 1a, ref. 8).

DISCUSSION

It is apparent that continuous light, hence photosynthetic activity, is not required for the actual diversion of ^{14}C into amino acids. A short period of white light at the time of normal dawn is sufficient to induce the response. In addition, the major response to the brief illumination is in the activity of NO3R. One peak (peak III) of NO3R activity clearly is related to the onset of illumination, and either in the normal photoperiod or in darkness following a brief illumination, activity reaches a maximum about 6 hr after the onset of the inducing light. If the photoperiod is given later than is normal for the population of plants then the NO3R activity develops later (Fig. 5) showing that the peak III of activity did not show entrainment to the normal light/dark cycle.

The induction of NO3R activity by a brief illumination with white or red light suggests the possibility of phytochrome control. It has been shown that phytochrome mediates the induction of NO3R activity in etiolated buds of pea (3) and the spectral characteristics of the induction in *Capsicum* warrant further investigation.

The *in vitro* NO3R assay used in this study does not allow an assessment of the *in vivo* activity of these activity peaks but the fact that the amino acid content of the leaves shows peaks in association with all three peaks of enzyme activity suggests that whether in the light or dark period, sufficient reducing power must be available for at least some of the potential NO3R activity to be realized in the production of amino acids. While the two peaks (peaks II and III) of NO3R activity that normally occur during the photoperiod could depend upon reducing power generated in the chloroplast, it is clear that the dark period peak (peak I) must be dependent upon respiratory activity for NADH, in a way similar to NO3R in nongreen tissues.

The absence of the early morning peak (peak II) when the preceding photoperiod is replaced by darkness or 20 min light (Fig. 4), suggests that development of the peak is dependent upon photosynthesis during the previous photoperiod. Unpublished results suggest that there is some entrainment to the normal light/dark timing, because retarding the previous dawn by 4 hr did not shift the peak noticeably. Part of the induction process may be set in operation by the dawn light-on signal but, perhaps, not until about 20 hr later is another constraint lifted and induction manifested in a measurable increase in NO3R activity (peak II).

The existence of the three separate peaks of NO3R activity raises the question of their location within the cell, or perhaps in different tissues within the leaf. It is tempting to speculate

that the peak normally manifests in the photoperiod (peak III), may be more closely associated with photosynthetic systems than either the peak occurring in the dark period or the early morning peak. Lips and Avissar (5) reported a nitrate reductase associated with the peroxisomes in tobacco leaves while Dalling *et al.* (1) could find only a cytosolic NO3R from leaves of the same species. This discrepancy may reside in the dominance of different NO3R in the two materials sampled, corresponding perhaps to the light peak and the early morning peak in *Capsicum annuum*.

The existence of the three separate peaks also raises the possibility of different factors being involved in their induction. It is clear that the light requirement of peak III is different from those of the other two peaks. Similarly, the role of the substrate and hormonal control may well differ in degree, at least, in the induction of the different peaks.

The demonstration of the three separate peaks of NO3R activity in leaves of *Capsicum annuum* cv. California Wonder makes it a suitable material for further investigation of different forms of NO3R, especially with regard to the conditions necessary for induction. The periodicity in activity reported here explains the periodicity in the nature of photosynthetic products. It seems clear that the periodicity in the availability of NH_4^+ in the leaf caused by the rhythms in NO3R activity (and nitrite reductase?) results in the activation and deactivation of pyruvate kinase (8) which controls the fate of carbon from the reductive pentose phosphate cycle. Other control points may exist but this direct influence of NO3R activity on photosynthetic carbon metabolism remains a major control factor in expanding leaves of *Capsicum annuum*.

Acknowledgments—I thank Mr. W. E. Bellew for assistance throughout the study and Mr. M. L. Higgins for the nitrogen determinations. Useful correspondence with Dr. W. S. Hillman (Brookhaven National Laboratory) is also acknowledged.

LITERATURE CITED

1. Dalling, M. J., N. E. Tolbert, and R. H. Hageman. 1972. Intracellular location of nitrate reductase and nitrite reductase. I. Spinach and tobacco leaves. Biochim. Biophys. Acta 283: 505–512.
2. Hewit, E. J. and D. J. D. Nicholas. 1964. Enzymes of inorganic nitrogen metabolism. *In:* K. Paech and M. V. Tracey, eds., Modern Methods of Plant Analysis, Vol. 7. Springer-Verlag, Berlin. pp. 67–110.
3. Jones, R. W. and R. W. Sheard. 1972. Nitrate reductase activity: phytochrome mediation of induction in etiolated peas. Nature New Biol. 238: 221–222.
4. Kannangara, C. G. and H. W. Woolhouse. 1967. The role of carbon dioxide, light and nitrate in the synthesis and degradation of nitrate reductase in leaves of *Perilla frutescens* L. New Phytol. 66: 553–561.
5. Lips, S. H. and Y. Avissar. 1972. Plant-leaf microbodies as the intracellular site of nitrate reductase and nitrite reductase. Eur. J. Biochem. 29: 20–24.
6. Martin, P. 1973. Nitratstickstoff in Buschbohnenblattern unter dem Gesichtspunkt der Kompartimentierung der Zellen. Z. Pflanzenphysiol. 70: 158–165.
7. Steer, B. T. 1973. Diurnal variations in photosynthetic products and nitrogen metabolism in expanding leaves. Plant Physiol. 51: 744–748.
8. Steer, B. T. 1974. Control of diurnal variations in photosynthetic products. I. Carbon metabolism. Plant Physiol. 54: 758–761.
9. Wallace, W. and J. S. Pate. 1965. Nitrate reductase in the field pea (*Pisum arvense* L.). Ann. Bot. 29: 655–671.
10. Youngberg, H. W., D. A. Holt, and V. L. Lechtenberg. 1972. Diurnal variation in nitrogenous constituents of alfalfa (*Medicago sativa* L.). Agron. J. 64: 288–291.