RHYTHMICITY IN NITRATE REDUCTASE ACTIVITY IN WHEAT EMBRYOS DURING GERMINATION

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SUMMARY

Nitrate reductase (NR) activity, induced by germination in KNO_3 showed a strong rhythmicity during the early stages of germination of wheat embryos. Peaks of activity occurred approximately every 12 h. There was no relationship between the amounts of nitrate in the embryos and enzyme activity. The fluctuations in enzyme activity did not appear to be due to reversible inactivation of the enzyme. Some evidence suggests that the fluctuations in enzyme activity may result from a more rapid degradation of enzyme during the decreasing phase of the rhythm.

INTRODUCTION

A rhythmicity in the activity of nitrate reductase (NR) has been reported in a number of plants (Hageman, Flesher and Gitter, 1961; Wallace and Pate, 1965; Harper and Hageman, 1972; Upcroft and Done, 1972; Steer, 1973; Cohen and Cumming, 1974; Bakshi, Farooqui and Maheshwari, 1978, 1979; Robin, 1979; Lewis, Watson and Hewitt, 1982). The reasons for these rhythmic fluctuations are not well understood. Our studies on the formation of NR activity during the germination of wheat embryos indicated that there was an initial phase of about 20 h, both in continuous white light and darkness, during which NR activity did not appear (Disa, 1981). In this communication, we confirm this finding and show that a strong rhythmicity in NR activity follows this initial phase with peaks occurring at 32, 44 and 56 to 60 h both in continuous white light and in continuous darkness.

MATERIALS AND METHODS

Wheat seeds (*Triticum aestivum* var. HD 2009) of uniform size were selected and spread on absorbent paper in glass Petri dishes; either water or 60 mM KNO_3 was added. Dishes were incubated at 25 °C in darkness or in light from cool white fluorescent tubes (visible light energy flux, 12 Wm^{-2}). For enzyme extraction, 20 embryos were dissected out from the seeds, washed with ice-cold distilled water, blotted dry gently and ice-chilled until homogenization. The embryos were homogenized in 2 ml of 50 mM phosphate buffer, pH 7·5, containing 3 mM EDTA and 3 mM cysteine. The homogenate was centrifuged at 15 000 r min^{-1} for 30 min at 4 °C and the supernatant used for the enzyme assay (Gupta, Sopory and Guha-Mukherjee, 1979), protein estimation (Lowry *et al.*, 1951) and for the determination of nitrate content (Wooley, Hicks and Hageman, 1960). Enzyme activities are expressed as nmol nitrite produced mg^{-1} protein h^{-1} and the accumulation of nitrate in the tissue is expressed as μmol of nitrate mg^{-1} protein.
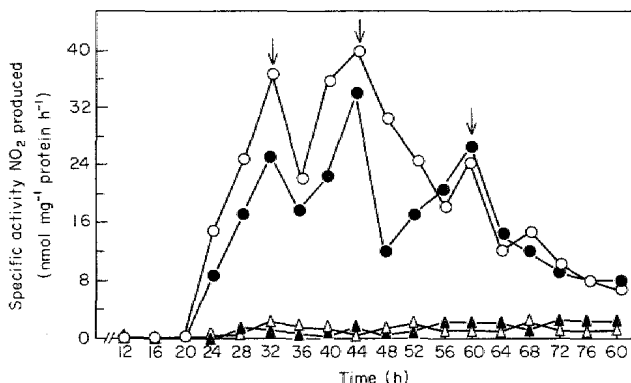
Fig. 1. Effect of light and darkness on NR activity. Seeds were germinated in KNO_3 under continuous light (○); or in darkness (●). Water controls were also run under continuous light (△) and in darkness (▲). At 4-h intervals embryos were dissected out and NR assayed.

RESULTS AND DISCUSSION

The presence of a phase of about 20 h, during which NR activity cannot be induced, has been reported in the initial stages of embryo germination of barley (Gupta *et al.*, 1979) and of rice (Ray, 1979). A similar non-inducible phase of 20 h duration was observed during wheat embryo gemination (Disa, 1981). A detailed study of the time course of NR activity, in embryos from seeds germinated in water (control) or in KNO_3 (60 mM) under continuous white light, revealed that there were peaks of enzyme activity occurring at 32, 44 and 56 to 60 h in the embryos from seeds supplied with KNO_3 but not from those germinated in water (Fig. 1). Similar rhythmic variations in NR activity were observed when the seeds were germinated in darkness (Fig. 1). Therefore, it is clear that NR activity is inducible by nitrate and that activity shows a strong endogenous rhythm with peaks approximately every 12 h.

There are reports where rhythmicity in NR activity has been shown to be correlated with similar fluctuations in uptake and availability of nitrate in the tissue (Wallace and Pate, 1965; Steer, 1973; Robin, 1979). To find out whether a similar correlation exists in wheat embryos the amount of nitrate and NR activity was measured at different time intervals over a period of 60 h germination. The nitrate content of the tissue was found to increase more or less linearly while NR activity showed the usual rhythms (Fig. 2). Therefore fluctuations in the uptake and availability of nitrate do not appear to be the cause of the observed rhythmicity in enzyme activity; the same is true for *Wolffia* (Bakshi *et al.*, 1979). Another possibility is that nitrate reductase becomes inactivated by reduction; such inactivation can be overcome by the addition of an artificial oxidant such as ferricyanide (Solomonson, 1974). However, when enzyme extracts were treated with $K_3Fe(CN)_6$ (250 μmol) for 10 min at 0 to 4 °C prior to enzyme assay, the usual rhythm in activity was still observed (Fig. 3). Therefore, rhythmicity in NR activity does not appear to be due to reversible inactivation and activation of the enzyme. A third possibility is that the rhythmicity results from the enzyme decaying at different rates at different times. To investigate this possibility the
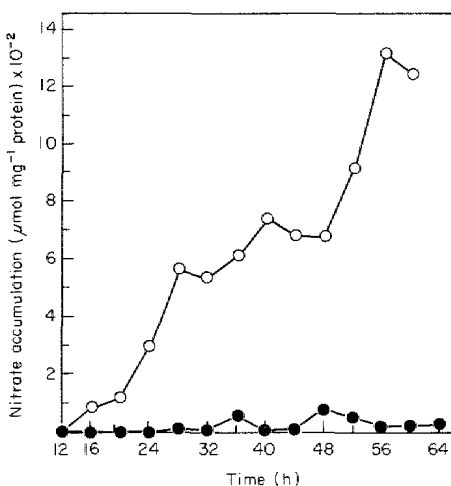
Fig. 2. Nitrate accumulation during seed germination. Seeds were germinated in water (●) or KNO$_3$ (○) under continuous light. At 4-h intervals, embryos were dissected out and nitrate estimated.
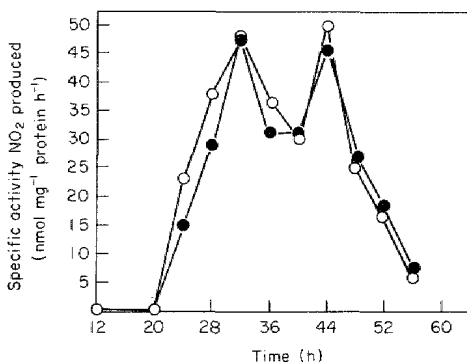

Fig. 3. Effect of K$_3$Fe(CN)$_6$ on NR activity. Seeds were germinated in KNO$_3$ under continuous light at 25 °C. At 4-h intervals, embryos were dissected out and enzyme extracts prepared. 250 μmol of K$_3$Fe(CN)$_6$ was added to the extract (2 ml) which was kept at 0–4 °C for 10 min. Thereafter, NR activity was assayed. Control without K$_3$Fe(CN)$_6$ (○); with K$_3$Fe(CN)$_6$ (●).

decay of the enzyme activity was measured both *in vivo* as well as *in vitro* from the 'increasing phase' and the 'decreasing phase' of enzyme activity.

To study the *in vitro* decay, enzyme extract was prepared from 24-h-old embryos ('increasing activity' phase) and 32-h-old embryos ('decreasing activity' phase), and kept at 0 and 30 °C; NR was assayed every 3 h over a period of 12 h. The half-life of the enzyme activity ($t_\frac{1}{2}$) from 'increasing' and 'decreasing activity' phases was found to be 5·4 and 3·4 h respectively at 0 °C [Fig. 4(a)]. However, there was no significant difference in the $t_\frac{1}{2}$ of the activities for enzymes kept at 30 °C; these were 2·1 h for the 'increasing' and 2·2 h for the 'decreasing activity' phase [Fig. 4(b)].
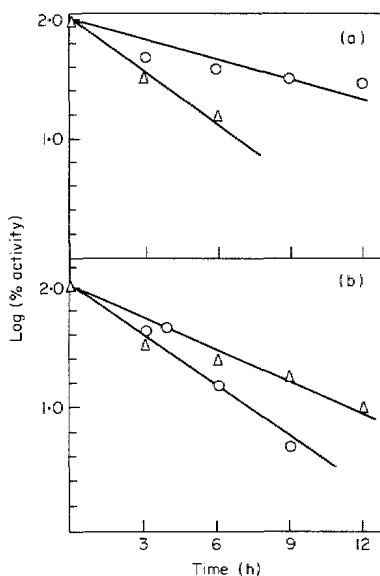
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Fig. 4 *In vitro* decay of NR activity. Seeds were germinated in KNO_3 for 24 or 32 h under continuous light. Embryos were dissected out and enzyme extract prepared. This was incubated at 0 °C (○) or 30 °C (△) and NR assayed at 3 h intervals. (a) 0–24-h-induced NR activity. (b) 0–32-h-induced NR activity.
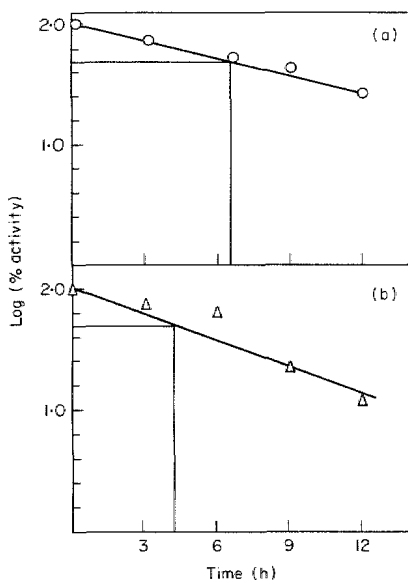

Fig. 5. *In vivo* decay of nitrate reductase activity. Seeds were germinated for 24 h or 32 h in KNO_3, under continuous light. The seeds were then transferred to sodium tungstate (3 mM) medium and allowed to grow further. At 3-h intervals, embryos were dissected out and NR was assayed. (a) 0–24-h-induced NR activity. (b) 0–32-h-induced NR activity.

To measure *in vivo* decay of enzyme, seeds were germinated in light for either 24 or 32 h in KNO_3 (60 mM) solution and then transferred to medium containing sodium tungstate (3 mM). NR activity was measured every 3 h over a 12 h period. The enzyme activity induced at 24 h was found to have a $t_{\frac{1}{2}}$ of 6·4 h while enzyme, induced at 32 h, had a $t_{\frac{1}{2}}$ of 4·2 h [Fig. 5(a), (b)]. From the above observations it appears that the enzyme from the decreasing phase may degrade faster than that from the increasing phase. Therefore, the endogenous rhythmicity of activity may result from oscillations in the degradation of NR enzyme protein (Cohen and Cumming, 1974). The observed rhythmic fluctuations in *in vitro* activity and stability of NR agrees with the report of Sherrard, Kennedy and Dalling (1979).

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