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REGULATION OF NITRATE REDUCTASE LEVEL IN PEA: IN VIVO STABILITY BY AMMONIUM

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SUMMARY: Ammonium, the end-product of nitrate-reduction, causes a marked increase in nitrate-dependent formation of nitrate reductase activity in pea shoot apices. The ammonium effect is mediated via a decrease in the rate of nitrate reductase decay. The increased stability of the enzyme in the presence of ammonium is indirect and depends upon protein synthesis. A regulatory role for ammonium-induced protein(s) is suggested.

The induction of NADH-nitrate reductase (EC 1.6.6.1), in algae (1-3) and fungi (4-6), is normally repressed in the presence of ammonium. In higher plants, however, the effect of ammonium on nitrate reductase induction has yielded varied results. Observations range from an inhibition (7-9) to promotion of induction (10-13). We recently reported that stimulation of NR activity by ammonium in pea shoot apices was indirect and not due to an increase in protein synthesis in general (14). In view of the fact that NR undergoes a rapid turnover (15), we wanted to know whether ammonium-mediated increase in NR activity was due to an increased rate of synthesis or due to a decrease in the rate of decay, both of which would result in an increase in enzyme activity.

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Abbreviation: NR, nitrate reductase.

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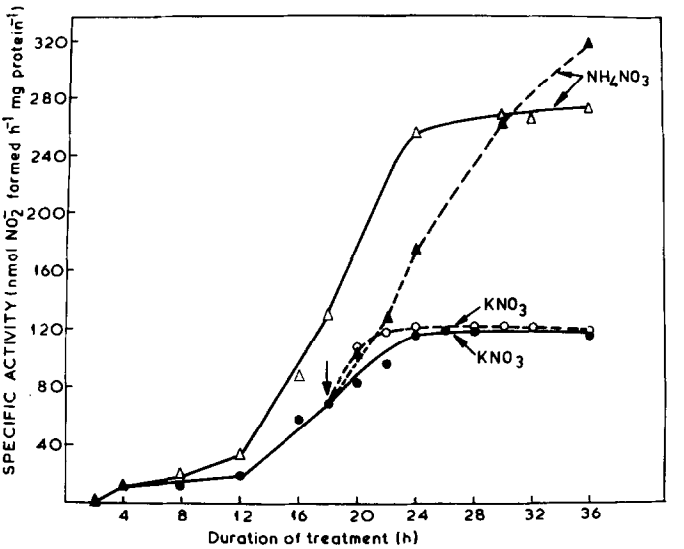

Figure 1. Time-course of nitrate reductase induction. Shoot apices were incubated with 20 mM KNO_3 or NH_4NO_3. Arrow indicates the transfer of shoot apices to fresh KNO_3 or NH_4NO_3 solutions.

MATERIALS AND METHODS

Pea (Pisum sativum L. cv. Bonneville) seedlings were grown in complete darkness at 25±1 C as described previously (14). Shoot apices (1 cm) excised from etiolated seedlings were induced in 5 ml of 20 mM KNO_3 or NH_4NO_3 (pH 5.8) containing 20 µg/ml streptomycin in white light (9.8 W.m-2, cool fluorescent) at 25±1 C. For enzyme assay about 0.5 g tissue was homogenised in a mortar and pestle at 4 C in 2 ml of 50 mM Tris-HCl buffer, pH 8.5, containing 3 mM EDTA. The homogenate was centrifuged at 13000 X g for 20 min and nitrate reductase activity was measured as described previously (14). For studies on decay of nitrate reductase activity under non-inducing conditions, the shoot apices were transferred to 5 ml of 20 mM KH_2PO_4 or $NH_4H_2PO_4$ (pH 5.8) containing 3 mM tungstate.

RESULTS

The results in Fig. 1 show the kinetics of nitrate reductase induction in both KNO_3 and NH_4NO_3 media. In NH_4NO_3 there was a marked increase in the rate of enzyme accumulation but there was no difference in the overall pattern of kinetics. The appearance of NR showed a lag and then a rapid rate of increase in the

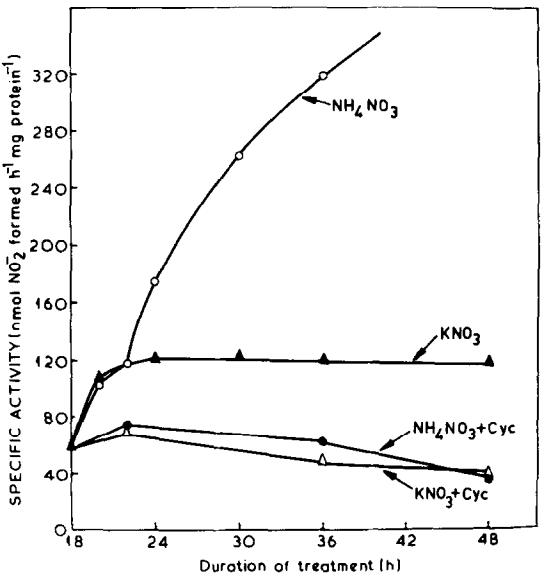

Figure 2. Effect of cycloheximide on the kinetics of nitrate reductase induction. Shoot apices were incubated in 20 mM KNO_3 for 18 h and then transferred to 20 mM KNO_3 or NH_4NO_3 containing 200 ug/ml cycloheximide.

enzyme activity before reaching a steady-state. During the phase of rapid increase in enzyme activity, the rate of enzyme accumulation in NH_4NO_3 was more than 2-fold higher than that in KNO_3. When shoot apices incubated in KNO_3 for 18 h were transferred either to KNO_3 or NH_4NO_3, the effect of ammonium became manifested after a lag of about 4 h. The lag period was followed by a striking increase in the rate of enzyme accumulation in NH_4NO_3 over KNO_3 control. Since individual amino acids and amino acid mixture (casein hydrolysate) do not replace ammonium requirement (14), the presence of lag phase for the manifestation of ammonium effect may represent the time taken for the synthesis of ammonium-induced protein(s). This was tested by supplying cycloheximide along with ammonium during the phase of rapid increase in enzyme activity. Cycloheximide (200 µg/ml), which caused about 60 per cent inhibition of nitrate-dependent formation

of nitrate reductase (14), resulted in a complete loss of ammonium -mediated increase in NR (Fig. 2). These results show that (1) ammonium mediated stimulation of NR does not reflect a general effect of ammonium on the enzyme turnover and (2) that the ammonium effect is dependent on protein synthesis.

Since NR undergoes a rapid turnover (15), the increased rate of NR accumulation in the presence of ammonium could be either due to an increased rate of enzyme synthesis or due to a decreased rate of enzyme decay. Since ammonium does not act by an increase in RNA and protein synthesis in general (14), a study for the possible role of ammonium on NR decay was carried out. Decay of NR activity was followed by blocking NADH-NR formation by 3 mM tungstate which was found to inhibit more than 95 per cent NR activity in the shoot apices. For studying the rate of loss of NR under inducing conditions, shoot apices induced in KNO_3 for 18 h were transferred either to KNO_3 or to NH_4NO_3 media containing 3 mM tungstate. The presence of ammonium in the medium markedly retarded the loss of activity. In NH_4NO_3 more than 50 per cent activity was left at the end as compared to only 10 per cent in KNO_3 (Fig. 3). Ammonium, thus, appeared to act by slowing down the decay rate of NR.

A further study on the rate of enzyme decay was carried out under non-inducing conditions. For this NR was induced both in KNO_3 (KNO_3-NR) and NH_4NO_3 (NH_4NO_3-NR) and the decay of the enzyme formed in each condition was followed in the presence of KH_2PO_4 as well as $NH_4H_2PO_4$. As seen in Fig. 4, the rate of loss of KNO_3-NR in KH_2PO_4 ($t_{0.5}$ 5.5 h) was much faster than in $NH_4H_2PO_4$. However, it may be noted that the ammonium effect became evident only after 3-4 h. Essentially similar results

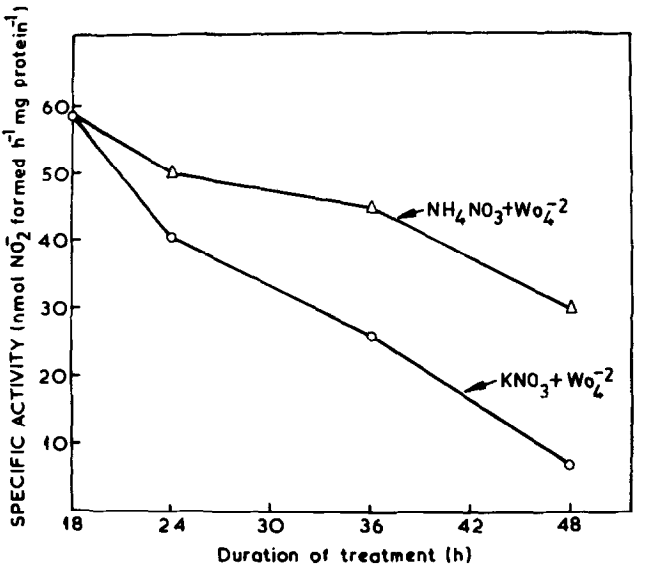

Figure 3. Decay of nitrate reductase under non-inducing conditions. Shoot apices were incubated in 20 mM KNO_3 for 18 h and then transferred to 20 mM KNO_3 or NH_4NO_3 containing 3 mM WO_4^{-2}.

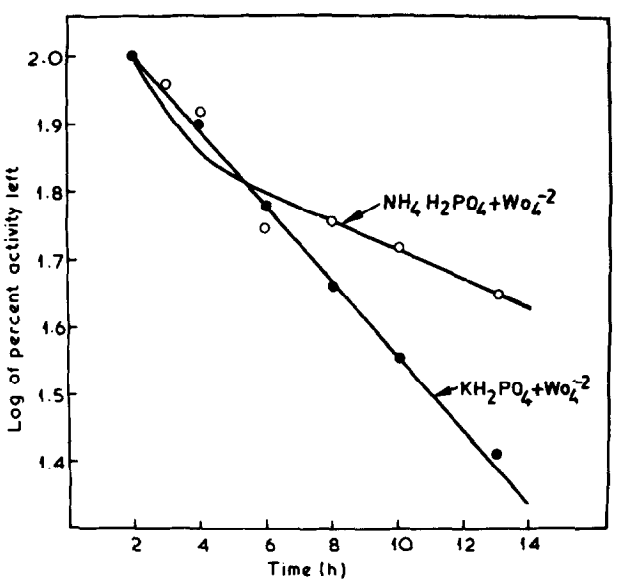

Figure 4. Decay of KNO_3-NR under non-inducing conditions. Shoot apices were incubated with 20 mM KNO_3 for 24 h, washed and incubated with 20 mM KH_2PO_4 for 2 h and then transferred to 20 mM KH_2PO_4 or $NH_4H_2PO_4$ containing 3 mM WO_4^{-2}.

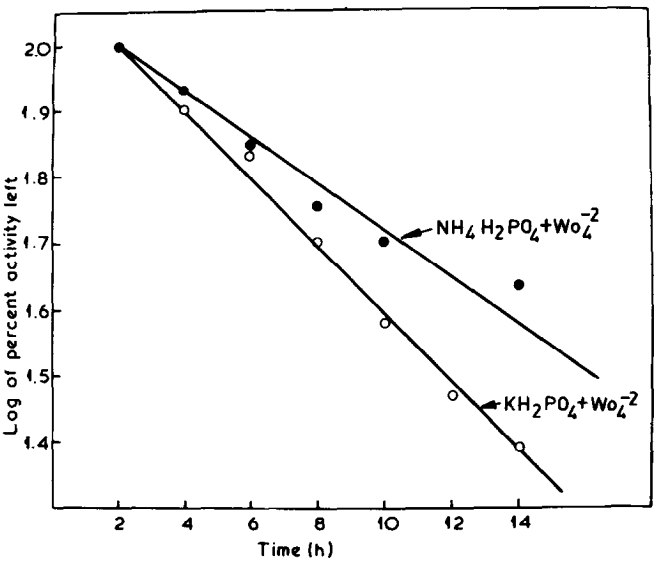

Figure 5. Decay of NH_4NO_3-NR under non-inducing conditions. Shoot apices were incubated with 20 mM NH_4NO_3, washed and incubated with 20 mM $NH_4H_2PO_4$ and then transferred to 20 mM KH_2PO_4 or $NH_4H_2PO_4$ containing 3 mM WO_4^{-2}.

were obtained when decay of NH_4NO_3-NR was followed (Fig. 5). The rate of loss of NR in the presence of $NH_4H_2PO_4$ was much slower ($t_{0.5}$ 8.5 h) as compared to that in the presence of KH_2PO_4 ($t_{0.5}$ 5.8 h). However, *in vitro* degradation rate of KNO_3-NR and NH_4NO_3-NR was similar ($t_{0.5}$ 3 h).

DISCUSSION

We reported earlier that the ammonium-mediated increase in NR activity is not due to an increase in RNA and protein synthesis in general (14). The data presented here show that the presence of ammonium in the induction medium causes *in vivo* stability of nitrate reductase. It may be emphasized, however, that this loss of detectable enzyme activity may merely reflect a reversible inactivation or inhibition rather than irreversible degradation of the enzyme. However, the reports on the presence

of NR specific inactivating enzyme in several plants including pea (16-18) suggest an irreversible loss of enzyme activity. We, therefore, conclude that ammonium acts by slowing down the decay rate of the enzyme.

The loss of ammonium mediated increase in NR activity by cycloheximide suggest that ammonium effect is dependent upon protein synthesis, perhaps upon the expression of a specific gene(s). De novo synthesis of proteins in response to ammonium in higher plants including pea has been shown (14, 19). We, therefore, suggest that ammonium-induced protein(s) may play a regulatory role in controlling the level of NR. It is not difficult to envisage the degradation of nitrate reductase being responsive to structural or conformational changes of the enzyme. The ammonium-induced protein may cause conformational change of nitrate reductase that would affect the availability of the peptide bonds susceptible to proteolytic enzymes. However, we have, at the moment, no explanation as to what physiological advantage(s) such a phenomenon would confer upon the plants.

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