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Comparative Studies on the Induction and Inactivation of Nitrate Reductase in Corn Roots and Leaves[1]

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ABSTRACT

A comparison of induction and inactivation of nitrate reductase and two of its component activities, namely $FMNH_2$-nitrate reductase and NO_3^--induced NADH-cytochrome *c* reductase, was made in roots and leaves of corn (*Zea mays* L. var. W64A × 182E). The three activities were induced in parallel in both tissues when NO_3^- was supplied. $WO_4^=$ suppressed the induction of NADH- and $FMNH_2$-nitrate reductase activities in root tips and leaves. The NO_3^--induced NADH-cytochrome *c* reductase activity showed a normal increase in roots treated with $WO_4^=$. In leaves, on the other hand, there was a marked superinduction of the NO_3^--induced NADH-cytochrome *c* reductase in the presence of $WO_4^=$.

The half-life values of NADH-nitrate reductase and $FMNH_2$-nitrate reductase measured by removing NO_3^- and adding $WO_4^=$ to the medium, were 4 hours in root tips and 6 hours in excised leaves. Addition of NO_3^- in the induction medium together with $WO_4^=$ gave partial protection of NADH-nitrate reductase and $FMNH_2$-nitrate reductase activities in both root tips and leaves with a $t_{0.5}$ of 6 and 8 hours, respectively. NO_3^- also reduced the loss of nitrate reductase activity from mature root sections. In the presence of cycloheximide, both NADH-nitrate reductase and NO_3^--induced NADH-cytochrome *c* reductase activities were lost at similar rates in root tips. NO_3^- protected the loss of NO_3^--induced NADH-cytochrome *c* reductase to the same extent as that of NADH-nitrate reductase.

Nitrate reductase is a complex enzyme which reduces NO_3^- to NO_2^- using NADH as the electron donor. Two component activities which give estimates of different portions of the enzyme complex are NO_3^--induced Cyt *c* reductase and a flavin mononucleotide nitrate reductase. The induction of NADH-NR[4] and the two associated enzyme activities have been studied in *Neurospora crassa* (16) and barley leaves (24). Similar studies on NADH-NR from root tissue have yet to be performed.

Nitrate reductase from several plant species has a relatively high turnover rate *in vivo* (14, 20, 25). The enzyme from corn leaves (14) and roots (11) disappears rapidly, with a half-life of 3 to 4 hr, under noninducing conditions. Turnover of NR has been definitively studied by Zielke and Filner (25), who used a density labeling technique. A simpler, but less accurate approach has been to use a specific inhibitor which stops the synthesis of an active enzyme complex. We have used tungstate ($WO_4^=$) and cycloheximide in our studies to inhibit the formation of a functional NR. $WO_4^=$ acts by replacing Mo in the $FMNH_2$-NR component of the enzyme. Thus the enzyme synthesized in the presence of $WO_4^=$ has lost its capacity to catalyse the reduction of NO_3^- by NADH and $FMNH_2$ (24). Cycloheximide, which should affect both components of the nitrate reductase enzyme, has been used in previous studies (14, 19, 20).

The objectives of the present study were 3-fold: *(a)* to compare the induction of NADH-NR, $FMNH_2$-NR, and NO_3^--induced NADH-CR in corn leaves and roots; *(b)* to compare the effect of $WO_4^=$ on the induction of NO_3^--induced NADH-CR in leaves and roots; and *(c)* to study the rates of decay of the three activities under inducing and noninducing conditions.

MATERIALS AND METHODS

Seedling Growth. Seeds of *Zea mays* L. (var. W64A × 182E), supplied by the Warwick Seed Co. of Blenheim, Ontario, were grown for 64 hr at 27 C on 0.9% (w/v) NO_3^--free agar medium made up with one-tenth strength Hoagland solution containing an additional supplement (0.02 μg/ml) of molybdenum (11).

In experiments where the leaves were used, seeds were planted in a 1:1 mixture of sand and vermiculite contained in plastic containers and were watered daily with one-tenth strength Hoagland solution with or without 10 mM KNO_3. The seedlings were grown for 7 days at 26 C in continuous light of 12,000 lux and a relative humidity of 70%.

Induction Studies. Intact seedlings were used for the induction of the enzyme in roots. The procedure has been described previously (1). For the induction of the enzyme in leaves, 10 cm long secondary leaves were excised and placed with their base down in 20 ml of 10 mM KNO_3 with or without 200 μM Na_2WO_4. Ten leaves were used for each treatment. The induction was carried out at 26 C in light of 12,000 lux and 70% relative humidity.

Inactivation Studies. For inactivation studies in roots, the seedlings were transferred, after a 3-hr induction period, to the solutions containing the appropriate treatment in one-tenth strength Hoagland solution. They were incubated in the dark at 27 C for the required times.

For inactivation studies in the leaves, the tip 10 cm of the leaves, grown in 10 mM KNO_3 for 7 days, were excised and placed in 20 ml of 200 μM Na_2WO_4 with or without 10 mM KNO_3^-. The leaves were then placed in light or darkness at 26 C and 70% relative humidity.

At the appropriate time intervals, the root tips (0–10 mm) and mature root sections (25–35 mm) were excised and frozen in liquid N_2. Likewise the leaves were removed from the treatment solutions, and the basal 2 cm which had been submerged in solution were discarded. The remaining tip sections were frozen in liquid N_2.

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[4] Abbreviations: NADH-NR: nitrate reductase; $FMNH_2$-NR: flavin mononucleotide nitrate reductase; NADH-CR: cytochrome *c* reductase.

EXTRACTION PROCEDURE

Method A. The samples were extracted with cold mortar and pestle in 4 volumes of 0.1 M HEPES (pH 7.4) containing 0.5 mM EDTA and 5 mM cysteine. The extracts were centrifuged at 30,000*g* for 15 min, and the supernatant was used as the source of enzyme.

Method B. In some studies a special extraction procedure described by Wallace (23) for isolating NO_3^--induced NADH-CR was adopted. The extraction medium was 0.05 M HEPES containing 0.4 M sucrose, 0.1% (w/v) BSA, 0.5 mM EDTA, 0.1 mM $MgCl_2$, and 5 mM cysteine (pH 7.5). The extracts were centrifuged at 10,000*g* for 10 min. The supernatant was further centrifuged at 272,000*g* for 60 min. Nitrate reductase and associated component activities were recovered in the supernatant. The supernatant thus obtained was partially purified by precipitation with 40% $(NH_4)_2SO_4$ (14). The pellet was taken up in a known volume of 0.1 M HEPES (pH 7.4) containing 0.5 mM EDTA.

Assay Methods. NADH-NR was assayed as described previously (1, 18). $FMNH_2$-NR was assayed according to the method outlined by Wray and Filner (24) except that flushing the assay tubes with N_2 was unnecessary. NADH-CR was assayed spectrophotometrically (4) with a Gilford spectrophotometer equipped with a linear chart recorder (24).

Sucrose Density Gradient Centrifugation. The procedure of Martin and Ames (9) as adopted by Wray and Filner (24) was followed. Crude cell-free extract was layered on top of the gradient, which consisted of 4.4 ml of a linear 5 to 20 % (w/v) sucrose gradient layered over a cushion of 0.8 ml of 50% (w/v) sucrose. The sucrose solutions were buffered in 0.1 M potassium phosphate buffer (pH 7.5) containing 10 μM FAD and 1 mM EDTA. The gradients were centrifuged at 42,000 rpm for 15 hr at 2 C using a SW50L rotor and a Beckman L-2 ultracentrifuge. After centrifugation, the centrifuge tubes were punctured, and 20 fractions of 15 drops each were collected. About 70 to 80% of the enzyme activity layered over the gradient was recovered.

NO_3^- and Protein Determinations. NO_3^- from the crude cell-free extracts was assayed as described previously (1). Soluble protein was precipitated with 10% trichloroacetic acid, and the protein levels were determined by the method of Lowry *et al.* (8) using BSA, fraction V, as the protein standard.

RESULTS

Kinetics of Development of NADH-NR, $FMNH_2$-NR, and NADH-CR Activities in Root Tips. Both NADH-NR and $FMNH_2$-NR activities increased rapidly after a short lag period and reached a steady state level in 4 hr (Fig. 1). The appearance of NO_3-induced NADH-CR followed a similar pattern. In these experiments, the $FMNH_2$-NR activity was always about one-half of NADH-NR activity, and in mature root sections it was absent altogether. Wallace (21) stated that a component of crude extract precipitated by 55 to 75% $(NH_4)_2SO_4$ interfered in the assay of $FMNH_2$-NR by causing the disappearance of product NO_2^-. In the present investigation, when extracts were partially purified by precipitation with 40% $(NH_4)_2SO_4$, the $FMNH_2$-NR recovery did not improve.

Cytochrome *c* reductase was assayed after centrifugation on a sucrose gradient. The major band (1 in Fig. 2A), which sedimented to the 50% sucrose layer at the bottom of the gradient, was not affected by the presence of NO_3^- (Fig. 2B). There were also two lighter bands: band 2 which was induced by NO_3^- and band 3 which did not respond to NO_3^- additions (Fig. 2, A and B). NADH-NR and $FMNH_2$-NR showed only one peak on the gradient and this co-sedimented with band 2 of NADH-CR (Fig. 2A). When $WO_4^=$ was added to the induction medium in addition to NO_3^-, the appearance of NADH-NR and $FMNH_2$-NR

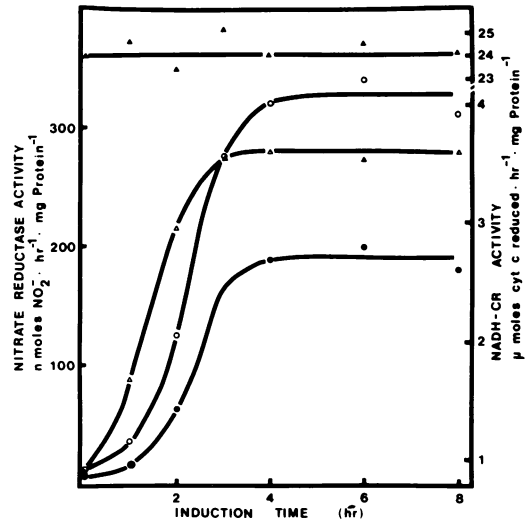

FIG. 1. Development of NADH-NR and its component activities in corn root tips. The roots were grown on NO_3^--free agar and induced in 5 mM NO_3^- solutions as described under "Materials and Methods." The root tips (0–10 mm) were harvested at the required time intervals and extracted by extraction method B. NADH-NR (○), $FMNH_2$-NR (●), NO_3^--induced NADH-CR (△), and constitutive NADH-CR (▲).

was inhibited. There was a normal appearance of bands 1, 2, and 3 of the NADH-CR (Fig. 2C).

Wallace (23) isolated the NADH-CR activity of band 1 from the enzyme activities of bands 2 and 3 by high speed centrifugation. He designated the NADH-CR activity retained in the pellet as constitutive NADH-CR, and the activity in the supernatant as NO_3^--induced NADH-CR. The NO_3^--induced NADH-CR activity also contains a constant but small fraction of constitutive NADH-CR activity represented by band 3 in Figure 2. In experiments where we have used this method, we have subtracted out this constitutive portion (Fig. 4; Tables III and IV).

Kinetics of Development of NR Activities in Leaves. When NO_3^- was supplied to the leaves, NADH-NR and its component activities increased over a period of 12 hr and then decreased slightly (Fig. 3, Table I). The induction process in leaves was much slower than in roots, but the final level of enzyme was much higher. In leaves the ratio of NADH-NR to $FMNH_2$-NR was near unity. It should be noted that leaves which had received no NO_3^- contained appreciable amounts of the three activities.

Effect of $WO_4^=$ on Induction of NR Activities. Addition of $WO_4^=$ to the induction medium gives an enzyme complex in which NO_3^--induced NADH-CR is induced while NADH-NR and $FMNH_2$-NR are inactive (24). In the present investigations, the induction of NADH-NR and $FMNH_2$-NR activities was completely inhibited by $WO_4^=$ in both root tips and leaves (Figs. 2C and 3). Similar levels of NO_3^--induced NADH-CR activity were obtained in corn root tips whether $WO_4^=$ was present or absent from the induction medium (Table I). In leaves, at steady state conditions, on the other hand, the level of induction of NO_3^--induced NADH-CR activity was two times higher when $WO_4^=$ was present in the induction medium. These results suggest that the regulation of the NO_3^--induced NADH-CR is different in roots and leaves.

Decay Kinetics of NADH-NR and Its Component Activities. Decay of NADH-NR and its component activities was studied in the presence of $WO_4^=$. Since $WO_4^=$ replaces Mo in the synthesis of new enzyme, its use permits one to follow the decay of the enzyme activity present at the start of the treatment (17). The data obtained were plotted on a first-order kinetics basis and half-life values were determined. A typical plot for the loss of enzyme activities from root tip sections is shown in Figure 4. The

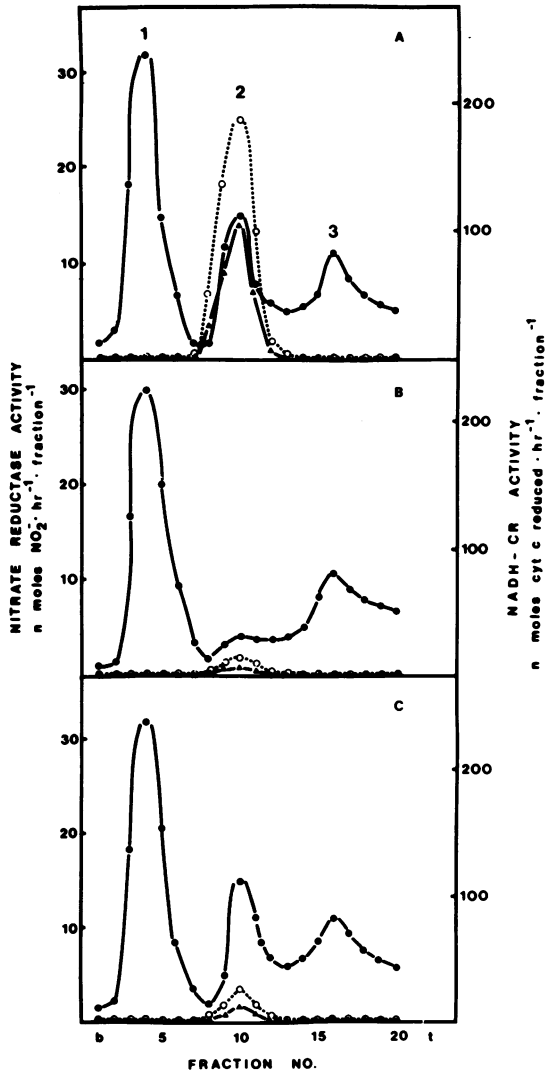

FIG. 2. Distribution of NADH-NR (○), $FMNH_2$-NR (▲), and NADH-CR (●) in corn root tip extract on a sucrose density gradient. Tissue supernatant (0.2 ml) was layered on top of the gradient and centrifuged at 42,000 rpm for 15 hr at 2 C using SW50 rotor in a Beckman L-2 ultracentrifuge. b (bottom) and t (top) of the gradient. A: Roots induced with 5 mM NO_3^-; B: roots treated with no NO_3^-; C: roots induced with 5 mM NO_3^- and 100 μM $WO_4^=$.

half-life values obtained for the various treatments are presented in Table II.

The results show that NADH-NR activity under noninducing conditions was lost rapidly from both mature root and root tip sections. As with earlier results (11), the enzyme from mature root sections was much less stable than the enzyme from root tip sections. The half-lives were 1.5 and 4 hr, respectively. $FMNH_2$-NR activity from root tips disappeared at a rate similar to that of NADH-NR. The half-life of NO_3^--induced NADH-CR from root tips was 7 hr. However, when $WO_4^=$ was replaced by cycloheximide the loss of NO_3^- induced NADH-CR was similar to that of NADH-NR (Table III).

Addition of 5 mM NO_3^- reduced the decay of the NADH-NR and $FMNH_2$-NR activities from root tips (Table II). The half-life values of the two activities were 6 hr in the presence of NO_3^-. With added NO_3^- the level of NO_3^--induced NADH-CR activity remained constant. In the cycloheximide treatment, NO_3^- reduced the loss of both the NO_3^--induced NADH-CR and NADH-NR (Table III). NO_3^- also reduced the decay of NADH-NR from mature root sections.

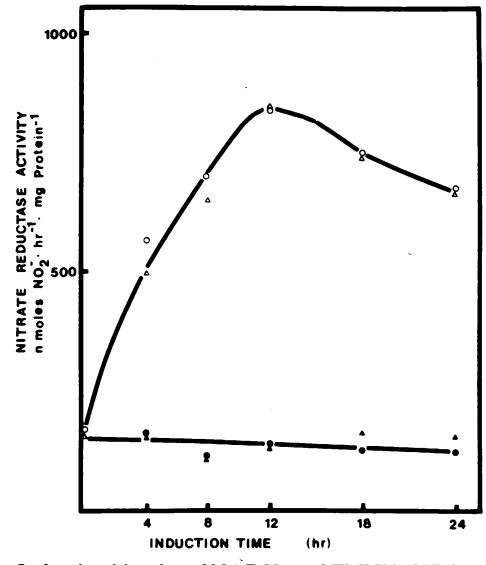

FIG. 3. Induction kinetics of NADH- and $FMNH_2$-NR in corn leaves. The seedlings were grown in NO_3^--free Hoagland solution as described under "Materials and Methods." After 7 days, the secondary leaves were excised and induced in 10 mM NO_3^- solution with or without 200 μM $WO_4^=$. The leaves were extracted by extraction method B. NADH-NR (○, ●), $FMNH_2$-NR (△ ▲). With closed symbols 200 μM $WO_4^=$ was included in the induction medium.

Table I. *Effect of $WO_4^=$ on Induction of NO_3^--induced NADH-CR Activity in Corn Seedlings*

The seedlings were grown and induced as described in Figs. 1 and 3. Enzyme was extracted according to method B.

Induction Time	Root Tips		Leaves	
	$-WO_4^=$	$+WO_4^=$	$-WO_4^=$	$+WO_4^=$
hr	*μmoles Cyt c reduced · hr⁻¹ · mg protein⁻¹*			
0	1.02	1.02	4.50	4.50
4	2.76	2.76	8.04	8.70
8	2.82	3.06	11.28	13.50
12	3.96	4.26	13.92	22.80
18			11.40	33.00
24	4.86	4.62	12.24	27.60

The decay kinetics of NADH-NR and $FMNH_2$-NR activities from leaves in light were also similar with half-life values of 6 hr (Table II). The loss of NO_3^--induced NADH-CR in the absence of NO_3^- had a half-life of 16 hr. As with root tips NO_3^- partially protected the decay of enzyme activities in leaves as well. With this treatment the half-life values for NADH-NR and $FMNH_2$-NR were 8 hr, and for NO_3^--induced NADH-CR 32 hr.

The enzyme activities in leaves were more stable in darkness than in light (Table II). NO_3^- had no effect in reducing the decay in darkness. The reason for this apparent difference in stability of the enzyme in light and dark is unknown.

DISCUSSION

The induction of nitrate reductase activity in higher plants in response to NO_3^- is well documented (3, 5). Wray and Filner (24) have shown further that in barley leaves both NADH-NR and its component activities, *i.e.* NADH-CR and $FMNH_2$-NR, are induced by NO_3^-. Dilworth and Kende (4) have also demonstrated a parallel induction of the three activities in *Agrostemma githago* embryo. The present results show that in both corn leaves and root tips NADH-NR and its component activities, $FMNH_2$-NR and NADH-CR, are induced when NO_3^- is supplied (Figs. 1 and 3; Table I). When $WO_4^=$ is added to the

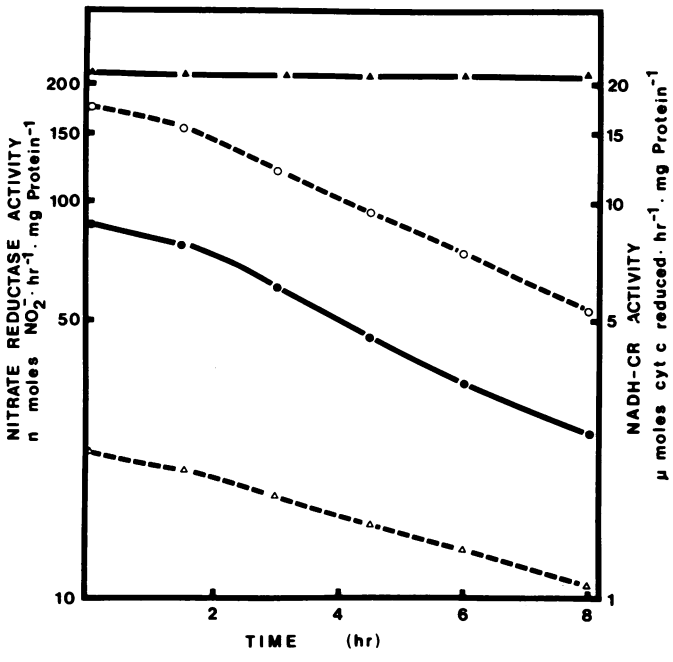

FIG. 4. Decay kinetics and NADH-NR and its component activities in corn root tips. Roots were grown and induced as described in Fig. 1. After 3 hr of induction in 5 mM NO_3^-, the roots were transferred to NO_3^--free solutions containing 100 μM $WO_4^=$. The roots were then harvested at different time intervals, and extracted according to method B. NADH-NR (○), $FMNH_2$-NR (●), NO_3^--induced NADH-CR (△), and constitutive NADH-CR (▲). In order to obtain the true rates of decay of NO_3^--induced NADH-CR, the enzyme activity formed in the tissue in the absence of NO_3^-, which could not be separated from NO_3^--induced NADH-CR by high centrifugation and was stable through out the inactivation period, was substracted from the values obtained and net activities were plotted.

Table II. *Half-life Values of NADH-NR, FMNH-NR, and NO_3^--induced NADH-CR under Inducing and Noninducing Conditions.*

The roots were grown and induced as described in Fig. 1. After a 3-hr induction period, the roots were transferred to the solution containing 100 μM $WO_4^=$ with or without 5 mm NO_3^- in one-tenth strength Hoagland solution. The leaves were grown as described in Fig. 3. After 7 days, the secondary leaves were excised and placed in the treatment solutions containing 200 μM $WO_4^=$ with or without 10 mM NO_3^- and placed in light or darkness. Enzyme was extracted according to method B. The enzyme activities were plotted on a first-order kinetics basis, and the half-life values were calculated.

Tissue	Treatment	Half-Live Values		
		NADH-NR	$FMNH_2$-NR	NADH-CR (NO_3^--induced)
			hr	
Root tip	$-NO_3^-$	4.0	4.0	7
	$+NO_3^-$	6.0	6.0	No loss
Mature root section	$-NO_3^-$	1.5		
	$+NO_3^-$	3.3		
Leaves (dark)	$-NO_3^-$	12.0	12.0	30
	$+NO_3^-$	12.0	12.0	30
Leaves (light)	$-NO_3^-$	6.0	6.0	16
	$+NO_3^-$	8.0	8.0	32

system, active NADH-NR and $FMNH_2$-NR are not produced in roots and leaves. In roots NO_3^--induced NADH-CR appears in amounts equal to the uninhibited control. In the leaves it appears in much higher amounts. A superinduction of NO_3^--induced NADH-CR was also seen in barley leaves by Wray and Filner

Table III. *Effect of Cycloheximide on Loss of NADH-NR and NO_3^--induced NADH-CR in Corn Root Tips*

The roots were grown and induced as described in Fig. 1. After 3 hr of induction, the roots were transferred to the treatment solutions containing 10 μg/ml cycloheximide with or without 5 mM NO_3^- in one-tenth strength Hoagland solution. The loss of enzyme activities was followed over a period of 8 hr. Extractions were made according to method B. Activities at 100% were NADH-NR, 220 nmoles of NO_2^- formed/hr·mg protein^{-1}; and NO_3^--induced NADH-CR, 2.53 μmoles of Cyt *c* reduced/hr·mg protein^{-1}.

Time	NADH-NR		NO_3^--induced NADH-CR	
	$-NO_3^-$	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$
hr		%		
0	100	100	100	100
4	63	77	59	69
8	46	55	43	53

(24). This observation suggests that in leaves the active form of the Mo moiety of the enzyme may in some way regulate the production of the active enzyme. When that portion of the enzyme is altered, the control on the production or turnover of nitrate reductase is altered in such a way that more nitrate reductase protein is made.

Nitrate reductase from corn roots (11) and leaves (14), has a high runover rate. The cessation of enzyme synthesis, therefore, leads to a rapid loss of enzyme activity. When $WO_4^=$ was used to stop the production of an active enzyme, NADH-NR and $FMNH_2$-NR were lost in parallel. The disappearance of NO_3^--induced NADH-CR was much slower, particularly when NO_3^- was present. When cycloheximide (Table III) or canavanine (unpublished results) were used to inhibit the production of the active enzyme, the three activities associated with nitrate reductase were equally affected.

In the present study nitrate reductase activity disappeared at a much faster rate from mature root sections than from root tips. In leaves the loss of nitrate reductase activity was two times faster in light than in darkness. In each case, NO_3^- reduced the rate of inactivation of the enzyme. This is particularly apparent for the NADH-NR and $FMNH_2$-NR in the presence of $WO_4^=$, and for NADH-NR and NO_3-induced NADH-CR in the presence of cycloheximide. Sorger *et al.* (17) and Subramanian and Sorger (19) have also observed that *in vivo* loss of nitrate reductase activities in *Neurospora crassa* is reduced when NO_3^- is present in the media. These observations suggest that in whole cells NO_3^- protects the enzyme from inactivation. Moreno *et al.* (10) have also shown that NO_3 protects the enzyme *in vitro* from inactivation by NADH.

Several mechanisms have been described which can account for the rapid loss of enzyme activity in response to changes in environmental conditions. These may be a specific (6) or nonspecific degradation (7, 13) of an active protein which would lead to an irreversible inactivation of the enzyme. In addition, there are reports of an inhibitory protein binding to an active protein which would lead to a reversible inactivation (2, 12). For the enzyme nitrate reductase, two mechanisms of inactivation have been reported: *(a)* an inactivating enzyme obtained from corn roots which specifically inactivates the NO_3-induced NADH-CR component of nitrate reductase (21–23), and *(b)* a reversible inactivation of the $FMNH_2$-component of the enzyme caused by oxidizing and reducing conditions (10, 15). Our results show that *in vivo* the two major components of the enzyme are equally sensitive to inactivation. Thus if the mechanisms described for *in vitro* systems are active *in vivo*, there must be some coordination which leads to a uniform inactivation of the entire enzyme molecule.

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