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Synthesis and Turnover of Nitrate Reductase in Corn Roots[1]

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ABSTRACT

The induction and reinduction of nitrate reductase in root tip or mature root sections show essentially a similar pattern: a lag, a period of rapid increase in enzyme activity and finally a period of relatively minor change. Both inductions are sensitive to 6-methylpurine and cycloheximide. Kinetic studies with 6-methylpurine suggest that the half-life of the messenger RNA for nitrate reductase in both sections is about 20 minutes. The rate of decay of nitrate reductase activity induced by transfer to a nitrate-free medium is slower in root tips ($t_{1/2}$ = 3 hours) than in mature root sections ($t_{1/2}$ = 2 hours). The enzyme from mature root sections is also less stable to mild heat treatments (27 C; 40 C) than the enzyme from root tip sections. The results indicate that factors regulating enzyme turnover show important changes as root cells mature and may be significant in determining steady state levels of the enzyme.

During early growth in maize seedlings, enzymes involved in the reduction of nitrate to ammonia may be considered luxury enzymes, since there is a rich source of amino acids supplied by the hydrolysis of endosperm proteins (13, 14). In addition, amino acids supplied by the endosperm are potential regulators of the embryo nitrate reductase (4, 7). Additions of nitrate to this system have no effect on the rate of hydrolysis of storage proteins (Oaks, unpublished), but do permit the induction of nitrate reductase in various parts of the embryo (Wallace and Oaks unpublished results).

In this paper we examine the details of the induction process in root sections. We have used root tip sections as models of populations of cells geared for growth (*i.e.*, protein and RNA synthesis) and more mature regions as models of cells where growth is no longer an important factor. Our results confirm earlier work on the requirement for RNA and protein synthesis (8, 17, 21) for the induction of nitrate reductase. In addition they show that as root cells age the enzyme becomes less stable.

MATERIALS AND METHODS

The seeds of *Zea mays* (Wf9 × 38-11) were surface sterilized by a brief treatment with Javex, a commercial bleach containing 6.0% NaOCl. They were planted under sterile conditions on nitrate-free 0.9% agar which contained one-tenth strength Hoagland solution and an additional supplement of molybdenum (0.02 μg/ml). The seedlings were grown for 56 hr at 26 C before transfer to nitrate induction media. At this time the primary root was approximately 5 cm long. Roots growing on the surface of the agar were selected. The nitrate induction solution contained 5 or 10 mM nitrate, levels which gave a good induction of nitrate reductase but which did not saturate the system either with respect to enzyme synthesis or accumulation of nitrate (Wallace and Oaks unpublished results). In addition, in long term experiments, these concentrations of nitrate did not inhibit growth of the primary root (Wallace and Oaks unpublished results). The inhibitor compounds tested were made up in solution just before use and the pH of the induction solutions was adjusted to 5.8. The experiments were conducted at 26 C in the dark. In all cases, intact seedlings were used and the primary root was sectioned only after the experimental treatment had been completed. The 0- to 10-mm and the 25- to 35-mm root sections were harvested at appropriate time intervals.

Extraction Procedure. Root tips and mature root sections were routinely frozen in liquid nitrogen, weighed, and stored at −20 C overnight. They were extracted with four volumes relative to weight of 50 mM phosphate buffer at pH 7.5. The buffer contained 0.5 mM EDTA and 5 mM cysteine. For enzyme preparations used in the *in vitro* inactivation, root tip sections were extracted as above, whereas the mature sections were extracted with two volumes of buffer. This had no effect on the specific activity of the enzyme, but insured that the protein levels in the two extracts were similar. The extracts were centrifuged at 30,000*g* for 30 min.

Assay Methods. The assay mixture for nitrate reductase was as follows: phosphate, pH 7.8, 90 μmoles; KNO_3, 20 μmoles; NADH, 1.1 μmoles and enzyme, in total volume of 1.5 ml. The colorimetric method for determining nitrite was essentially the same as that described by Sanderson and Cocking (15). A unit of nitrate reductase is defined as that amount of enzyme which can form 1 mμmole of nitrite in 1 hr.

Protein was determined by the Folin method (11) after precipitation with 10% trichloroacetic acid. Bovine serum albumin was used as the standard.

Chemicals. Rifamycin SV and rifampicin were a gift from Lepetit, Milan; Actinomycin D, a gift from Merck and Company, Rahway, New Jersey; and α-amanitin, a gift of Professor T. Wieland, Heidelberg, Germany. Cycloheximide, 8-azaguanine, and 6-methylpurine were purchased from Sigma Chemical Company, St. Louis, Missouri.

RESULTS

Time Course for the Induction of Nitrate Reductase in the Corn Root. The results in Figure 1 show the kinetics of the induction of the enzyme in the root tip and mature root sections. At zero time, a low but significant level of nitrate reductase was found in both regions of the root. After a half-hour, an increase in nitrate reductase level was observed in the tip region followed by a rapid increase in the level of enzyme

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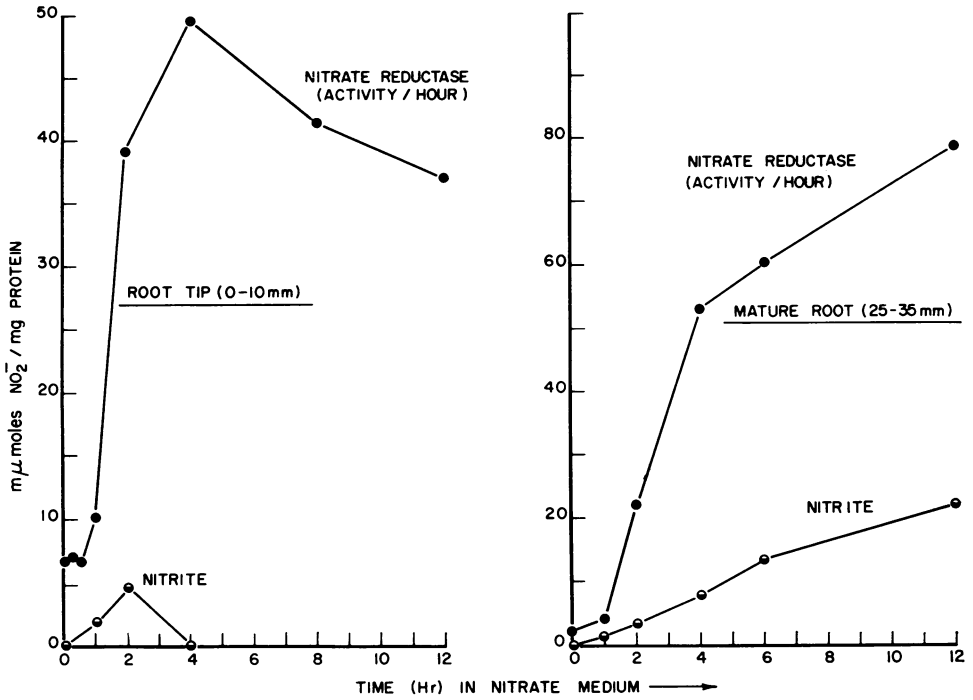

FIG. 1. Induction kinetics of nitrate reductase. Seedlings were grown on a minus nitrate agar for 56 hr at 26 C, and then transferred to a 10 mM nitrate-inducing medium. At the designated times the 10-mm root tips and 25- to 35-mm root sections, 50 per sample, were harvested and frozen immediately in liquid nitrogen. Subsequently, the sections were extracted as described in "Materials and Methods."

activity in the subsequent 2-hr interval (an increase of about 29 units/hr). Between 4 to 6 hr a maximum level of nitrate reductase was achieved in the root tip, and after that time there usually was a slight decline in the level of the enzyme. The leveling off of enzyme activity is probably not the result of nitrate limitation because (*a*) 70% of the original nitrate supplied to the root remained in the medium after 12 hr, and (*b*) transfer to a fresh nitrate medium at 4 hr resulted in only minor changes in enzyme level. During the induction there was a small transient accumulation of nitrite in the root tips which reached a peak at 2 hr, suggesting that nitrate reductase was the rate-limiting reaction after 2 hr.

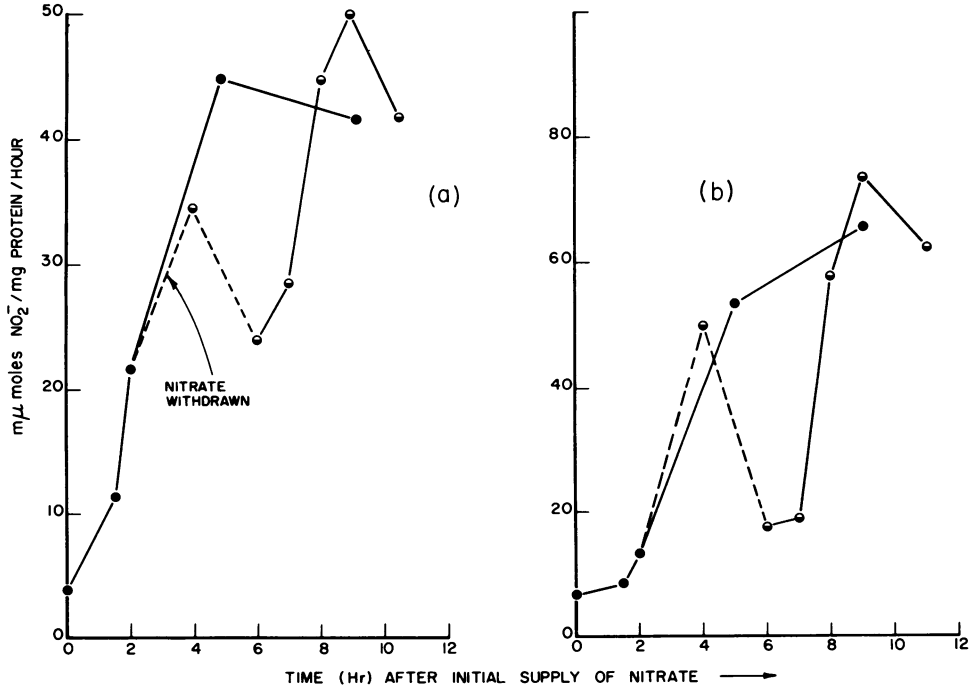

FIG. 2. Induction and reinduction of nitrate reductase. Seedlings were grown and induced, in this case with 5 mM nitrate as described in Figure 1. a: Root tips; b: mature sections. ●: Control induction; ◒: transfer to nitrate-free solution at 2 hr and transferred again to 5 mM nitrate at 6 hr.

Table I. *Influence of Inhibitors of RNA on the Induction of Nitrate Reductase in the Corn Root*

Seedlings were grown and induced, in this case with 5 mM nitrate, as described in Figure 1. In each case a 3-hr induction period was used. The control values were 43.8 and 23.1 nmoles nitrite/mg protein in 1 hr for root tip and mature sections respectively.

Inhibitor	Level Tested	Pretreatment with Inhibitor	Increase in Nitrate Reductase Level after 3-Hr Induction with 5 mM Nitrate (Control = 100)	
			Root tip (0–10 mm)	Mature root (25–35 mm)
		hr		
6-Methylpurine	0.5 mM	0	50	32
6-Methylpurine	0.5 mM	0.5	8	10
6-Methylpurine	0.5 mM	2	0	0
Actinomycin D	10 μg/ml	2	74	87
EDTA	0.1 mM	2	77	
EDTA + Actinomycin D	0.1 mM 10 μg/ml	0[1]	100	
EDTA + Actinomycin D	0.1 mM 10 μg/ml	1[1]	60	
EDTA + Actinomycin D	0.1 mM 10 μg/ml	2[1]	48	
8-Azaguanine	1 mM	2	100	
α-Amanitin	10 μg/ml	2	95	
Rifampicin	10 μg/ml	2	117	
Rifamycin	10 μg/ml	2	94	

[1] When EDTA was used together with Actinomycin D, the control value, with EDTA alone was 40.6 nmoles nitrite/mg protein in 1 hr.

In mature root sections the lag in induction of nitrate reductase was approximately 1 hr. The activity then increased at a rate of approximately 16 units of enzyme per hour for 1 to 4 hr and after that time at a much reduced rate of about three units per hr. There was a gradual and unexpected accumulation of nitrite in mature sections throughout the experiments. The same general trends were observed with 1, 5, and 25 mM nitrate (Stevens and Oaks, unpublished).

In a second type of experiment, seedlings were induced in a medium containing 5 mM nitrate for 2 hr and transferred to a nitrate-free medium. The initial induction of nitrate reductase in the root tip (0–10 mm) and a relatively mature root region (25–35 mm) was similar to that described in Figure 1. After the transfer to a nitrate-free medium, the increase of nitrate reductase was similar to the controls during the next 2 hr (Fig. 2). After this time, there was a marked decline in the level of the enzyme in the minus nitrate treatment. When the nitrate deprived seedlings were transferred back to a nitrate solution at 6 hr a reinduction of nitrate reductase occurred. In the tip region the lag, rate of increase in enzyme level, and the final level of enzyme were the same during the primary and secondary inductions. In the mature region there was a lag, of approximately 1 hr in both inductions before an increase in enzyme was observed. However, during the reinduction, the rate of increase of enzyme activity was two times that observed during the initial induction. Again the final level of enzyme attained during the reinduction was similar to that which would have been obtained had the roots been in nitrate continuously. These results suggest that the new enzyme formed during the reinduction is not an activation of previously synthesized enzyme but an actual new synthesis.

Influence of RNA Synthesis Inhibitors on the Induction of Nitrate Reductase in the Corn Root. The results in Table I illustrate the effect of various potential inhibitors of RNA synthesis on the induction of nitrate reductase in the corn root. In each case a 3-hr induction period was used. 6-Methylpurine supplied in the induction medium together with nitrate inhibited by 50 and 68% the potential synthesis of nitrate reductase activity in the root tip and mature root sections.

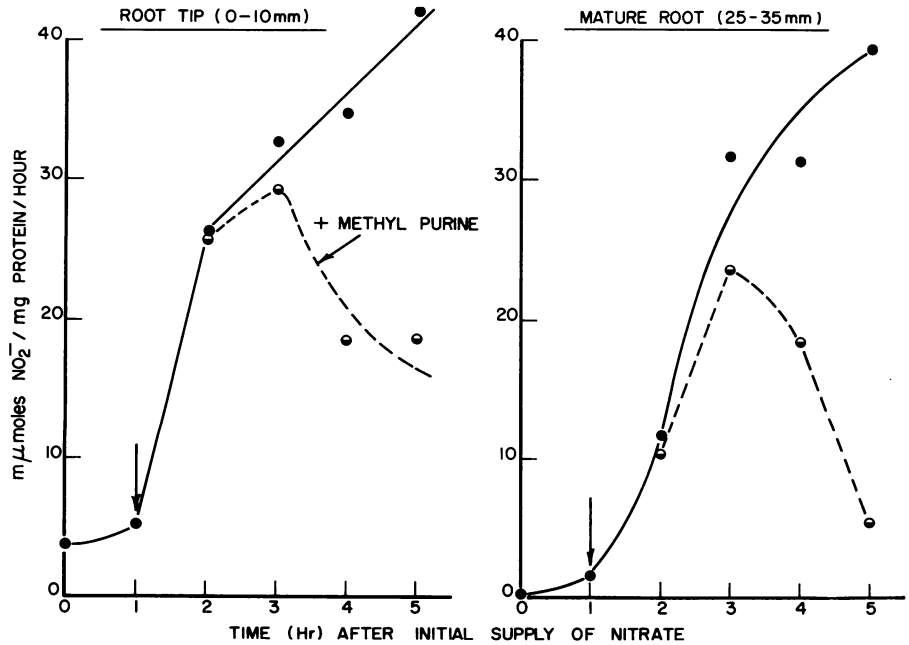

FIG. 3. Effect of 6-methylpurine on the induction of nitrate reductase. Seedlings were grown and induced, in this case with 5 mM nitrate as described in Figure 1. ●: Control induction; ◒: addition of 0.5 mM 6-methylpurine at arrow.

Pretreatment of the roots with 6-methylpurine for one-half or 2 hr prior to the addition of nitrate resulted in an almost complete inhibition of the increase in nitrate reductase. Thus a minimum of 30 min is required for sufficient penetration of the analogue to inhibit the induction of nitrate reductase. A second analogue, 8-azaguanine 1 mM did not inhibit the induction when added 2 hr prior to nitrate.

Actinomycin D at a final concentration of 10 μg/ml was found to be only slightly inhibitory when used alone. However, when added together with 0.1 mM EDTA, it caused a 40% inhibition of the induction. EDTA at this concentration had no real effect on the growth rate or the induction of nitrate reductase. α-Amanitin (10 μg/ml) which should inhibit enzyme inductions in eukaryote systems (2) had no effect on the induction of nitrate reductase. Rifamycin and its derivative rifampicin which specifically inhibit the initiation reaction of DNA dependent RNA polymerase in *E. coli*, but are ineffective in mammalian systems (6) also gave negative results in this system.

Since these results indicated that 6-methylpurine was effective in inhibiting the induction of nitrate reductase, the kinetics of the inhibition were studied in more detail. For these experiments, the seedlings were pretreated for 1 hr with nitrate and transferred either to fresh nitrate or to nitrate and 6-methylpurine (Fig. 3). During the initial 2-hr treatment with 6-methylpurine, the level of nitrate reductase was only slightly less than the control value. After this time, the enzyme showed a decay pattern similar to that obtained by withdrawing nitrate from the medium (Figs. 2, 4). Since 6-methylpurine is presumed to interfere with RNA synthesis, these results show that RNA synthesis is required for the induction of nitrate reductase. They also suggest that the preformed mRNA for nitrate reductase is exhausted in corn roots after a 2-hr interval.

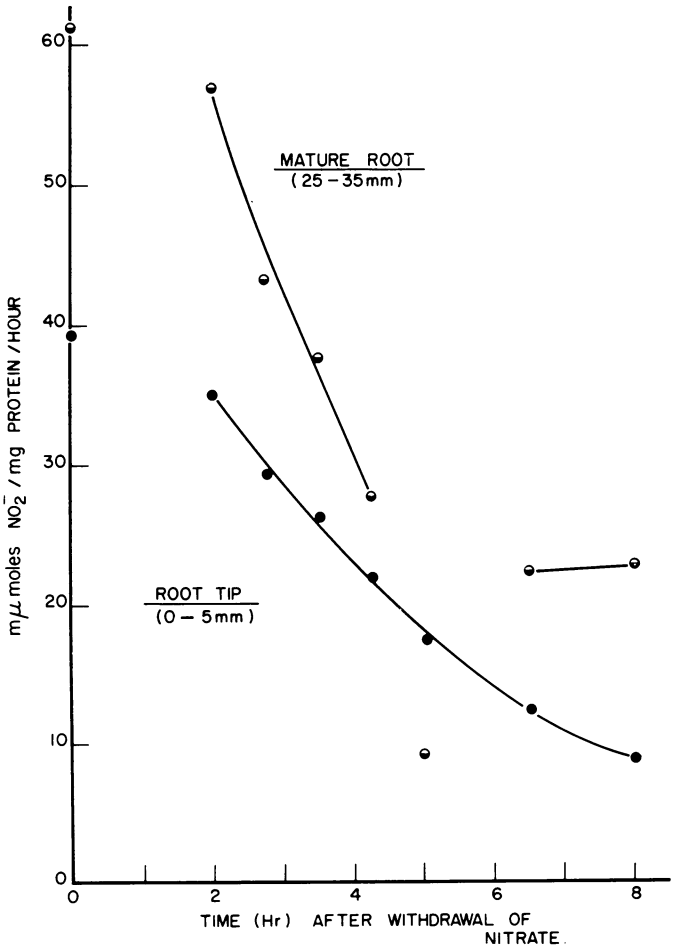

FIG. 4. Decay of nitrate reductase. Seedlings were grown and induced, in this case with 10 mM nitrate, as described in Figure 1. Induction time was 4 hr; subsequently, the seedlings were transferred to a nitrate free medium, and the roots were harvested at the appropriate times. ●: 10-mm root tips; ◒: 25- to 35-mm root sections.

Table II. *Effect of Time of Addition of 6-Methylpurine on the Induction of Nitrate Reductase*

Seedlings were grown and induced, in this case with 5 mM nitrate, as described in Figure 1. A concentration of 0.5 mM 6-methylpurine (MP) was used as indicated.

Initial Treatment	Transfer Treatment	Total Time in Nitrate	Specific Activity of Nitrate Reductase	
			Root tip (0–10 mm)	Mature root (25–35 mm)
		hr	*nmoles nitrite/mg protein·hr*	
A. Primary induction				
1 hr nitrate	None	1	5.5	3.0
	2 hr nitrate	3	33.3	35.3
	2 hr nitrate + MP	3	26.0	28.8
3½ hr nitrate	None	3.5	28.3	30.3
	2 hr nitrate	5.5	41.3	63.4
	2 hr nitrate + MP	5.5	38.9	51.5
5½ hr nitrate	None	5.5	45.0	56.9
	2 hr nitrate	7.5	66.8	77.1
	2 hr nitrate + MP	7.5	64.2	69.8
B. Reinduction				
2 hr nitrate + 4 hr minus nitrate	None	0[1]	19.1	18.3
	2 hr NO_3	2	39.1	63.6
	2 hr nitrate + MP	2	25.1	34.5

[1] Zero time in the reinduction experiment is time of second addition of nitrate.

A series of experiments were performed in which 6-methylpurine was added at various times after the addition of nitrate (Table II). After a further 2 hr in NO_3 and 6-methylpurine the seedlings were harvested. With the root tips there was a marginal inhibition of nitrate reductase in each case. These results show that there is no differential effect of 6-methylpurine on enzyme synthesis during the course of induction. In addition during the reinduction, there is still a requirement for RNA synthesis which suggests synthesis of nitrate reductase protein rather than a reactivation of pre-existing protein.

Factors Controlling the Turnover of Nitrate Reductase. The characteristics of the decay pattern for nitrate reductase were examined in root tip and mature sections. In these experiments an induction period of 4 hr preceded the transfer to a minus nitrate medium. After the initial transition period of 2 hr, seedlings were sacrificed at regular intervals. An exponential loss of enzyme was evident for both sections (Fig. 4). The break in the decay pattern for the mature sections after 5 hr is reproducible and could be due to some reinduction by nitrate released from storage pools. When the data from 2 to 4.5 hr

after withdrawal of nitrate was plotted on a semilog scale the loss of nitrate reductase in the root tip and mature sections was linear. The t½ values were calculated to be 3 hr for the tip sections and 2 hr for the mature sections.

Stability of the Enzyme to Heat Inactivation. The results in Figure 4 suggest that the enzyme from mature sections is less stable than the enzyme from root tips. To check this point, further extracts were prepared for both root tip and mature sections. Preliminary experiments showed that the rate of decay was enhanced by dilution of the enzyme particularly with treatments of 27 and 40 C. Accordingly the volume of buffer used in the extraction was adjusted so that similar amounts of protein were present during the inactivation (about 0.8 mg/reaction). This had no effect on the initial specific activities of the enzyme. The rate of decay of activity at 0 C, 27 C, or 40 C is more rapid in extracts from mature sections (Fig. 5). Thus in both types of experiment (Figs. 4, 5), the results suggest that the stability of the enzyme decreases as the root cells age.

Effect of Cycloheximide on Synthesis and Degradation of Nitrate Reductase. Preliminary results with cycloheximide showed that concentrations of 5 μg/ml or greater caused a 100% inhibition of enzyme formation when added together with nitrate. With 1 μg/ml there was a 50% inhibition of the induction. Cycloheximide inhibited further increases in nitrate reductase activity in both root tip and mature root sections when added at 1, 2, or 3 hr after the addition of nitrate or during the reinduction period. The effects of 10 μg/ml cycloheximide on the development of enzyme activity in root tip sections are shown in Figure 6. The decay rates in the presence of cycloheximide are essentially the same as when nitrate is removed from the medium. Thus cycloheximide appears to inhibit the synthesis of the enzyme but to have no effect on its degradation.

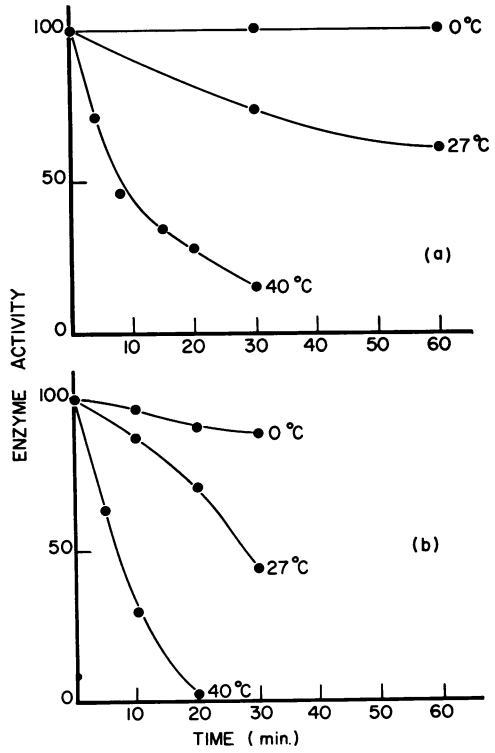

FIG. 5. Effect of heat denaturation on the decay of nitrate reductase *in vitro*. Seedlings were grown and induced, in this case with 10 mM nitrate, as described in Figure 1. After a 4-hr induction period, the root tips and mature sections were frozen in liquid nitrogen and then extracted and assayed as described in "Materials and Methods." Protein levels were 0.8 mg/reaction. a: 100 is 77.4 mμmoles/hr for the root tips; b: 70.4 for the mature sections.

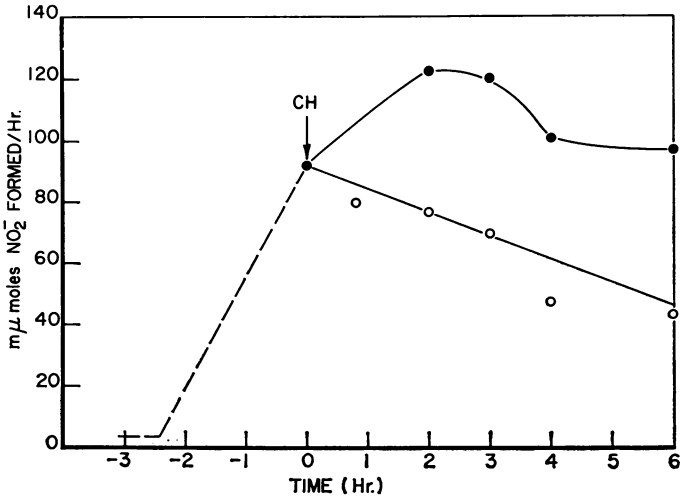

FIG. 6. Effect of cycloheximide on the loss of nitrate reductase. Seedlings were grown and induced, in this case with 10 mM nitrate, as described in Figure 1. After a 3-hr induction period, the seedlings were transferred to fresh 10 mM nitrate (●), or to a medium which contained 10 μg/ml cycloheximide in addition to other additives (×).

DISCUSSION

In the induction of nitrate reductase in root tip and mature sections there is a lag, a period of rapid increase, and a period of little change. As with other inducible enzymes in eukaryote systems (5, 16, 17, 20, 21, 24), this pattern of induction suggests the involvement of activating and inactivating systems which are responsive to both the level of co-inducer, in this case nitrate, and repressor, in this case unknown. In fungi where genetic and biochemical features have been examined in detail several unlinked genetic loci are responsible for the structure and induction properties of nitrate reductase (1, 18, 19). In higher plants the enzyme is equally complex and hence we assume that several genes are important here too. Recently, Warner *et al.* (22) have identified two genetic components in *Zea mays* which are important in determining the steady-state level of nitrate reductase. One results in a high level of enzyme which we take to be part of the genetic complex involved in the synthesis of the enzyme. The other gives an unstable enzyme and hence may be an integral part of a specific inactivating system.

In agreement with earlier work (8, 17, 21), our results show that both RNA and protein synthesis are important in the induction. In addition, the main features of this system are similar in both regions of the root. One difference is that half an hour is required for the development of protein synthesis machinery in root tip sections, whereas 1 hr is required in mature sections. We take the longer lag time and the slower rate of increase of enzyme activity in the mature root sections as an indication of a more sluggish biosynthetic machinery, since previous studies have indicated higher rates of macromolecule synthesis in the meristematic regions of the root (3, 9, 13). The faster rate of enzyme formation during the reinduction in the mature sections does, however, represent a real difference which will be investigated further.

During the reinduction one would expect an immediate increase in enzyme activity if an activation of previously synthe-

sized protein or mRNA were involved. In fact as in the primary induction, we find lag times of one-half and 1.5 hr for root tip and mature sections during the reinduction. This suggests that pre-existing mRNA and protein is lost from the system during the 4-hr nitrate-free interval. Results with 6-methylpurine are consistent with this, since each induction is sensitive to the analogue. After 2 hr with 6-methylpurine the rate of loss of enzyme parallels that observed in the nitrate withdrawal experiments (Figs. 2, 3, 4) in both root tip and mature root sections. Of this 2 hr, one-half hr is required for adequate penetration of the analogue. Thus the $t_{1/2}$ of the nitrate reductase mRNA is about 20 min.

The decay of a number of inducible enzymes is sensitive to inhibitors of RNA and protein synthesis (20, 21, 24). Tomkins *et al.* (20) have examined this point most critically. The addition of actinomycin D to their system late in the induction period results in an overproduction of tyrosine aminotransferase (20). This suggests the presence of an inactivating system which is more sensitive to actinomycin D than is the aminotransferase induction system. It also suggests some degree of specificity in the inactivating system. More recently, Tomkins' group have shown in synchronous cultures of hepatoma cells that the inactivating system appears later in time in response to the inducer (12). In a similar type of experiment, we added 6-methylpurine at different times in the induction and reinduction of nitrate reductase (Table II). The results showed no differential effect. Thus, if two systems each requiring RNA and protein synthesis are active in establishing the steady-state level of the enzyme they have similar turnover rates.

In all systems examined nitrate reductase has a high rate of turnover (17, 19, 21, 23), and corn roots are no exception to this general pattern. However, consistency of results with different systems is not apparent after this observation has been made. In barley leaves, for example, cycloheximide prevents the normal dark loss of nitrate reductase (21), whereas in corn leaves (17) and corn roots (Fig. 6), it has no effect. In fungi, nitrate stabilizes the enzyme against degradation when cycloheximide is present in the system (19). Ammonia, on the other hand, brings about the rapid loss of the enzyme (10, 19). Such clear-cut effects of NO_3 and NH_4^+ have not been reported in higher plants and in fact may not be present. Zielke and Filner (23), for example, found that the presence of nitrate in the medium had no effect on the turnover of the enzyme in tobacco cells.

Our results show that the inactivation is more rapid in enzyme prepared from mature regions of the root. Similarly heat denaturation is more effective in extracts prepared from mature root cells. This may represent the development of a specific inactivating system or simply an altered physical-chemical stability of the enzyme. The important point here, however, is that both types of experiment indicate an increased instability of the enzyme as the root cells age.

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