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Studies on the Induction of Nitrate Reductase by Nitrite in Bean-Seed Cotyledons

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Nitrate reductase of sterilized bean seed cotyledons is more readily induced by nitrite (10 mM) then by nitrate (100 mM) when incubated in these solutions for 24 h. Inhibition of induction by tungstate, dependence on NADH and presence in extracts of endogenous nitrate at effective substrate concentrations were observed with both inducers. Nitrite was oxidized to nitrate after absorption but the concentration produced thereby was less than 5% of that produced by direct absorption of nitrate from the incubation medium. Nitrite may be a direct inducer of nitrate reductase in bean seed cotyledons.

The induction of nitrate reductase by nitrate in plants has been demonstrated by Tang and Wu [11], Cabdela *et al.* [1] and Hageman and Flesher [2]. Sometimes nitrate reductase may be induced by either nitrate or nitrite: this has been found in *Neurospora* (Nicholas *et al.* [8]), cauliflower (Candela *et al.* [1]) and radish seedlings (Ingle *et al.* [5]). Joy [6], on the contrary, reported that nitrite did not induce nitrate reductase in *Lemna minor*. The manner, however, in which nitrite causes the induction of nitrate reductase has not been investigated. Various possibilities are indicated: (a) nitrite may induce the enzyme directly, its efficiency as an inducer varying between tissues; (b) nitrite is oxidized to nitrate in the tissue of the growth medium, the nitrate then being responsible for the induction of the enzyme; (c) nitrite allows the activation of a nitrate-reducing enzyme different from nitrate reductase; (d) nitrate reductase activity induced by nitrite is an artefact.

The present studies were undertaken in order to test the different explanations advanced for the nitrite-induced nitrate reductase activity, using cotyledons of bean seeds. We had previously observed that nitrite brough about induction of the enzyme activity in bean seed cotyledons.

MATERIALS AND METHODS

Plant Materials

Seeds of bean, *Phaseolus vulgaris*, were imbibed in distilled water for 4 h at 30 °C in darkness. Thereafter, they were sterilized in 50% $HClO_4$ (v/v) for 10 min. The seeds were then thoroughly rinsed in about 2 liters of sterilized distilled water, after which the seed coats and embryos were removed. The remaining cotyledons were transferred in groups of five to Petri dishes (7-cm diameter) where they were placed on three layers of filter paper impregnated with 6 ml distilled water and 3 drops of chloramphenicol (0.5 mg/ml). The cotyledons were incubated under these conditions for 20 h at 30 °C in darkness.

Induction Procedure

Nitrate reductase was induced by subsequently incubating the cotyledons under identical conditions in an incubation medium containing KNO_3 or KNO_2 in given concentrations, 0.1 M phosphate buffer at pH 7.4, and chloramphenicol as previously indicated. When Na_2WO_4 was used, it was added to the induction medium.

The described procedure was carried out in a sterile room; sterilized solutions and glassware were used.

Homogenization

The cotyledons were ground with a pestle in an ice-cold mortar containing 0.05 M phosphate buffer pH 7.4, using 4 ml buffer per g tissue. The homogenate was passed through two layers of gauze and the filtrate was used for enzyme assays.

Enzyme Assay

Nitrate reductase was determined following essentially the method of Hewitt and Nicholas [4]. Excess NADH at the end of the reaction was removed

Enzyme. Nitrate reductase (EC 1.6.6.2).

according to the technique described by Medina and Nicholas [7] in order to obtain reliable values for nitrite. Nitrite content in the assay mixture was determined before and after reaction; enzyme activity was indicated by the increase in nitrite measured.

Nitrate was determined as described by Sloan and Sublett [10].

RESULTS

Induction of Nitrate Reductase by Nitrite

When bean seeds are placed in solutions containing KNO_3, nitrate reductase activity may be observed after an induction period of 24 h (Fig. 1). A similar effect is obtained, however, when KNO_2 replaces KNO_3 in the induction medium (Table 1). The levels of nitrate reductase activity observable after induction in the presence of nitrite are of the same order of magnitude as those obtained in the presence of nitrate. The possibility was examined that nitrite might be bound to some fraction in the homogenate and be slowly released during the assay. No evidence for nitrite release at 4 °C or 30 °C was found over 10-min intervals up to 60 min.

Characterization of the Nitrite-Induced Enzyme

We then considered the possibility that nitrite activated an enzyme other than nitrate reductase. Investigating this hypothesis, we compared several characteristics of the nitrate-reducing enzyme induced in the presence of nitrate and the nitrate-reducing enzyme appearing after exposure of the cotyledons to nitrite.

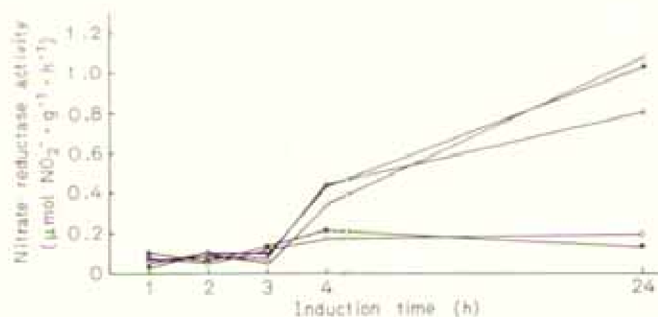

Fig. 1. *Nitrate reductase induction in different concentrations of KNO_3.* (●) H_2O; (○) 10 mM KNO_3; (□) 25 mM KNO_3; (△) 50 mM KNO_3 and (■) 100 mM KNO_3

Table 1. *Induction of nitrate reductase in the presence of KNO_3 or KNO_2*
Induction time, 24 h

Induction medium		Nitrate reductase activity
	Concn	
	mM	$\mu mol \times g^{-1} \times h^{-1}$
KNO_3	100	2.14
	2	0.30
KNO_2	5	1.02
	10	2.14
	20	4.00

Table 2. *Nitrate reductase dependence on NADH and NO_3^- after its induction in NO_3^- and NO_2^-*
Induction time, 24 h. 20 μmol KNO_3 and 0.8 mg NADH were used

KNO_3	NADH	Nitrate reductase activity in	
		100 mM KNO_3	10 mM KNO_2
		$\mu mol \times g^{-1} \times h^{-1}$	
+	+	2.45	2.22
—	+	1.92	2.06
+	—	0	0
—	—	0	0

Table 2 shows that the activity of the enzymes obtained with nitrate or nitrite have a very similar dependence on NADH although there is only slight dependence on added nitrate for crude extracts. It is possible that the source of the nitrite formed during the assay may not be nitrate, but the dependence of the reaction on NADH does not support this view. Alternatively, it may be that in both nitrate-induced and nitrite-induced tissue, nitrate is present in amounts capable of maintaining the rate of reduction allowed by the enzyme formed.

Nitrate and Nitrite Contents of Tissue

The nitrate content of nitrite-incubated tissue is shown in Table 3. It seems quite clear that a considerable amount of nitrite was oxidized to nitrate in the tissue and diffused out into the medium. The amount of nitrate present in cotyledons incubated in 10 mM KNO_2 seems to be just enough to support the reductive capability developed in the cotyledons.

Bacterial Contamination

Oxidation of nitrite to nitrate could be caused by bacterial contamination. This possibility was considered despite the fact that the seeds had been treated with $HClO_4$ immediately before initiation of

Table 3. *Nitrate content of cotyledons and induction media after induction of nitrate reductase in 10 mM KNO_2 for 24 h*
The nitrate content of the incubation medium was taken for 6 ml

Incubation media		NO_3^- in	
	Concn	Cotyledons	Incubation medium
	mM	μmol/g	μmol
H_2O		0.05	0.07
	5	0.11	2.32
KNO_2	10	2.23	2.48
	20	2.29	4.91

Table 4. *Nitrate levels in incubation medium from which cotyledons were withdrawn after 24-h induction*
Medium without cotyledons was incubated for the time periods indicated in table

Incubation time	Nitrate content in media of		
	H_2O	KNO_3	KNO_2
h	µmol/ml medium		
0	0.01	37.61	0.50
6	0.01	35.05	0.79
24	0.01	38.41	0.53

Table 5. *Inhibition of nitrate reductase induction by $WO_4^=$ in medium containing either KNO_3 or KNO_2 as inducers*
Induction time, 24 h

$WO_4^=$	Nitrate reductase activity in	
	100 mM KNO_3	5 mM KNO_2
mM	$\mu mol \times g^{-1} \times h^{-1}$	
0	1.65	0.34
0.1	0.99	0
0.5	0.40	0
1.0	0.06	0

Table 6. *Time-dependence inhibition of nitrate reductase by $WO_4^=$*
Inducer was supplied during the last 24 h of inhibition. The 48-h treatment consist of 24-h incubation in water and tungstate followed by 24 h of $H_2O + WO_4^= + KNO_2$ (10 mM)

$[WO_4^=]$	Time in inhibitor	Nitrate reductase activity	Inhibition
mM	h	$\mu mol \times g^{-1} \times h^{-1}$	%
0	0	1.74	—
0.5	24	1.78	0
0.5	48	1.57	10
1.0	24	1.29	26
1.0	48	0.35	80

incubation; the induction medium contained chloramphenicol and all the solutions and glassware had been carefully sterilized. Seeds were incubated, as in previous experiments, in KNO_2 for 24 h and then removed from the induction medium. The induction medium remaining was tested at varying intervals to see whether further oxidation of nitrite took place. Results are shown in Table 4. It is clear that oxidation of nitrite to nitrate was not discernable after removal of the cotyledons from the incubation medium.

The Effect of Tungstate

The induction of nitrate reductase by nitrate is inhibited by $WO_4^=$ as shown by Heymer *et al.* [3]. Table 5 shows that the induction of nitrate reductase by nitrite is also sensitive to tungstate. This fact constitutes further evidence of the similarity of the nitrate-reducing enzyme induced in the presence of either nitrate or nitrite. It should be pointed out that to observe adequate inhibition of the slowly permeating tungstate ions, sufficient time should be allowed for the ion to penetrate the tissue (Table 6).

Kinetics of Induction

Two feasible explanations now remain to account for the induction of nitrate reductase by nitrite. (a) Nitrite is oxidized to nitrate in the tissue and the nitrate formed induces nitrate reductase. It is noteworthy, however, that the levels of nitrate in the tissue are much lower when given 10 mM KNO_2 in the induction medium than when supplying 100 mM KNO_3; in both cases the levels of nitrate reductase induced are very similar (Tables 1 and 2). (b) Nitrite is a direct inducer of nitrate reductase.

These two possibilities were tested by following the appearance of nitrate and nitrate reductase in cotyledons incubated in nitrate or nitrite. The results are shown in Fig. 2.

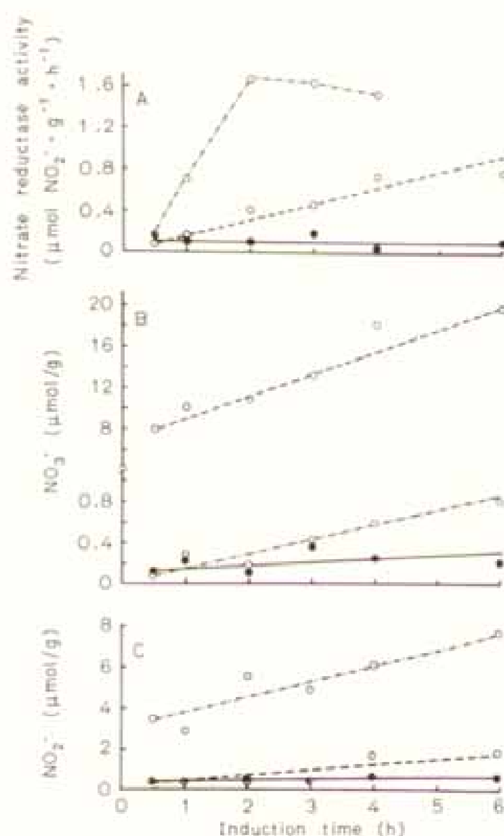

Fig. 2. *Rate of induction of nitrate reductase and tissue level of NO_3^- and NO_2^- in different media.* (A) Rate of induction of nitrate reductase after induction by different media; tissue level of (B) NO_3^- and (C) NO_2^- during incubation in presence of different media. (○— —○) 100 mM KNO_3, (○-- -- --○) 10 mM KNO_2 and (●——●) H_2O

DISCUSSION

Nitrite is much more effective than nitrate in inducing nitrate reductase in bean seed cotyledons. This induction does not seem to be the result of bacterial contamination. Significant release of loosely bound NO_2^- by fractions of the homogenate has not been detected.

The tissue appears to be capable of oxidizing NO_2^- to NO_3^- since nitrate appears in the tissue and the incubation medium containing nitrite. The results in Fig. 2 show several new facts: (a) nitrite is much more efficient than nitrate in the induction of the enzyme; 10 mM KNO_2 brings about a faster induction of nitrate reductase than 100 mM KNO_3; (b) nitrate formed in the presence of nitrite cannot be responsible for the induction of nitrate reductase. This conclusion is based on the fact that only a very small amount of nitrate is present in nitrite-induced tissue after 1 or 2 h, (Fig. 2B), while the induction of the enzyme is more than four-fold greater than in nitrate-incubation cotyledons. (c) The rate of induction of nitrate reductase is five-fold faster in nitrite than in nitrate. Furthermore, it seems that most of the induction of the enzyme in the presence of nitrite is completed during the first 2 h of induction.

Fig. 2C indicates that nitrite accumulates very slowly in nitrate-incubated seeds and this is accompanied by a comparably slow induction of nitrate reductase. When nitrite is supplied directly from the medium, however, enzyme induction is much faster. One may assume, therefore, that the slow reduction of nitrate by the constitutive level of the enzyme causes a comparably slow accumulation of nitrite and, consequently, the sluggish rate of enzyme induction. Availability of nitrate is not limiting induction as may be seen in Fig. 1.

The rate of nitrate reduction may be also determined by the supply of reducing equivalents necessary for the reduction, as is the case in leaves of etiolated barley seedlings (Roth-Bejerano and Lips [9]).

Whatever the limitation of nitrate reductase induction by nitrate (activity of constitutive enzyme, supply of reducing power or both) such limitation does not seem to affect the induction of the enzyme by nitrite.

The levels of nitrate reductase after 24 h induction in nitrite (Tables 1 and 5) are quite variable. This is due to the fact that the level of the enzyme induced in nitrite starts falling, shortly after the two first hours of induction (Fig. 2A). After 24 h, one measures the remaining activity of the enzyme after induction of nitrite.

Nitrite may be, therefore, a direct inducer of nitrate reductase in bean seed cotyledons. This capability is not related to the extent of nitrite oxidized to nitrate in the tissue (Fig. 2B).

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