Phytochemistry, Vol. 25, No. 7, pp. 1563–1565, 1986.
Printed in Great Britain.

0031-9422/86 $3.00 + 0.00
Pergamon Journals Ltd.

THE INHIBITION OF NITRATE REDUCTASE INDUCTION BY SPERMINE IN EXCISED RADISH COTYLEDONS

S. K. SRIVASTAVA* and S. M. MUNGRE

Biochemistry Department, M.S. University, Baroda 390002, India

(*Revised received* 28 *November* 1985)

Key Word Index—*Raphanus sativus*; Cruciferae; radish; nitrate reductase; polyamines; nitrate assimilation.

Abstract—The induction of nitrate reductase in excised cotyledons of radish seedlings was inhibited by the polyamines, spermidine and spermine, in light and dark, but putrescine had no effect. Spermine had no effect on the uptake of nitrate or the stability of the enzyme, but inhibited the synthesis of the enzyme.

INTRODUCTION

Nitrate reductase (NR) is widely distributed, occurring in all organisms capable of utilizing nitrate, including bacteria, fungi and higher plants. It is the first enzyme of the nitrate assimilatory pathway regulating the amount of nitrate nitrogen available to the plant for the synthesis of amino acids. NR is an inducible enzyme, nitrate serving as the inducer in most plants [1]. The induction of NR is a protein-synthesis dependent response [2]. Nitrate is also essential for the maintenance of the enzyme level which declines on removal of nitrate from the medium [3].

We have reported earlier [4] the effect of polyamines on the enzymes of nitrogen assimilation pathway during germination of radish seeds in presence of nitrate. NR and glutamine synthetase (GS) were inhibited in light but not in dark grown seeds. Since NR is induced by nitrate in excised tissue also, an attempt was made in the present study to investigate the mechanism of inhibition of NR by polyamines using excised cotyledons.

RESULTS AND DISCUSSION

When the excised cotyledons were pretreated with polyamines for 30 min before initiating the induction of NR in either light or dark, putrescine had no effect on NR, but the polyamines, spermidine and spermine, inhibited NR activity (Table 1). Spermine was more effective than spermidine. This is in contrast to our earlier observation [4] where polyamines were found to inhibit only the light mediated increase in NR activity when the seeds were grown in presence of nitrate. In dark-grown seeds polyamines had no effect. The reason for this difference is not clear, though it does not seem to be associated with the removal of embryonal axis since treatment of the whole seedling instead of excised cotyledons also showed a similar effect. Thus the long term effect during growth in the dark appears to differ from the short term effect during induction for 3 hr. Since spermine was more effective, further studies were carried out using spermine as the representative compound.

Table 1. Effect of polyamines on the induction of nitrate reductase activity in excised radish cotyledons.

Pretreatment	Nitrate reductase activity % of control	
	Light	Dark
—	100*	100*
Putrescine	98	98
Spermidine	65	60
Spermine	36	30

Cotyledons from 36 hr old dark-grown seedlings were excised and pretreated with the test compounds (1 mM) for 30 min before induction with KNO_3 (10 mM) in light or dark for 3 hr.

*NR activity in the control group was 1.3 and 1.1 units/g fr. tissue in light and dark respectively.

The level of NR induced in a tissue depends upon the amount of nitrate available within the cell. Nitrate uptake is an energy dependent process requiring the hydrolysis of ATP [5]. It is suggested that nitrate may induce the synthesis of a nitrate-specific permease [6]. Further, NR in higher plants is known to have a relatively fast turnover and various mechanisms may operate to inactivate or degrade the enzyme synthesized in response to nitrate. The NR activity obtained at any given time is thus a result of the balance between the synthesis and degradation processes. Polyamines may inhibit NR activity in the cell by affecting one or more of these processes.

Since polyamines are known to bind to membranes and affect their permeability [7] the effect of spermine was studied on the uptake of nitrate at high and low concentrations of ambient nitrate, in dark and light. However, the nitrate remaining in the medium at the end of the induction period did not differ in the absence or presence of spermine (data not shown) indicating that spermine did not inhibit NR activity by inhibiting the uptake of nitrate. Spermine also had no effect on NR when added to the assay system.

Nitrate in the cell may be present in more than one pool, a larger storage pool, inaccessible to reduction and a

*To whom correspondence should be addressed.

Table 2. Effect of spermine on nitrate reductase, metabolic pool of nitrate and nitrate content of excised radish cotyledons

Period of induction (hr)	Pre-treatment	Nitrate reductase activity (units/g fr. tissue)	Nitrate content (μmol/g fr. tissue)	Metabolic pool of nitrate (units/g fr. tissue)
0	—	0.01	0.0	—
1	—	0.51	1.05	—
	+ Spermine	0.22	1.73	—
2	—	0.79	1.44	0.020
	+ Spermine	0.36	2.57	0.008
3	—	1.19	2.08	0.028
	+ Spermine	0.47	2.66	0.013

Cotyledons from 36 hr old dark-grown seedlings were excised and pretreated with spermine (1 mM) for 30 min before induction with KNO_3 (10 mM) in the dark for 3 hr. Nitrate reductase, nitrate content and the metabolic pool of nitrate were measured at a specified period of induction.

smaller 'metabolic pool' which is responsible for the induction of NR activity. Nitrate present in the metabolic pool is affected by a number of factors such as light and the rate of its uptake. The size of the metabolic pool in the cell may thus be a factor regulating the nitrate assimilation [8]. Though spermine did not affect the uptake of nitrate from the medium, it may alter the intracellular redistribution of nitrate and thus the size of the metabolic pool.

The effect of spermine was, therefore, studied at different intervals of induction in relation to NR activity, nitrate content of the tissue as well as the metabolic pool of nitrate. The metabolic pool of nitrate was decreased by 50% in spermine treated tissue (Table 2) and the nitrate content of the tissue was greater in spermine-treated than in the control group. Spermine may have thus altered the size of the metabolic pool in the cell, resulting in a decreased NR activity. Alternatively, the lowered metabolic pool as measured by the *in vivo* nitrate reduction may reflect an inhibited NR activity in spermine-treated tissue leading to an increased accumulation of nitrate. Since spermine did not affect the enzyme activity when added to the assay *in vitro*, it may be affecting the stability and/or synthesis of NR.

The effect of spermine was studied on the stability of NR during storage, both *in vitro* and *in vivo*. *In vitro* stability was studied by adding spermine to the homogenate during storage. The rate of decay of the enzyme did not differ in the absence or presence of spermine and there was a loss of *ca* 40% in control as well as in the spermine-treated group at the end of 6 hr of storage (data not shown).

Spermine, however, brought about a faster decrease in the level of NR in both light and dark when added during storage *in vivo* (Fig. 1). Light increased the activity of NR in the control till the 2nd hr, compared to the spermine-treated group. The rate of decline in the control group was faster in dark than in light and after 10 hr there was *ca* 40% decrease in NR activity in the cotyledons stored in the dark compared to the light control. In the spermine-treated group, however, there was a progressive decrease in NR level in cotyledons stored in either light or dark. However, the rate of decline of the enzyme from the 2nd

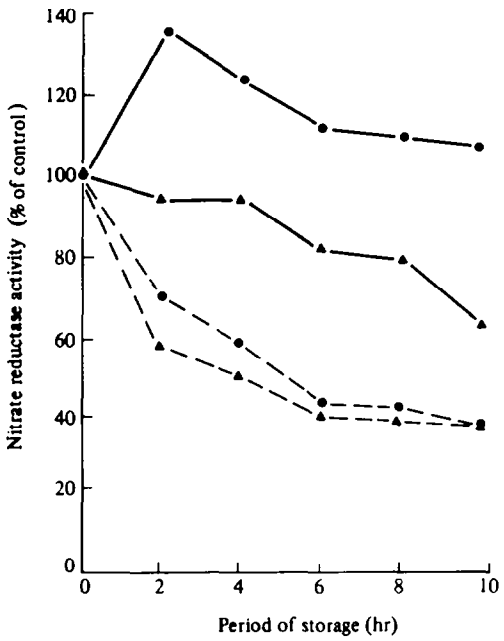

Fig. 1. Effect of spermine on the stability of nitrate reductase *in vivo*. Cotyledons from seeds grown in the dark for 36 hr were excised and NR was induced with KNO_3 (10 mM) in the dark for 3 hr. Cotyledons were then washed with distilled water and stored in light (●) or dark (▲) in the absence (—) or presence (- - -) of spermine (1 mM). NR activity was assayed at specified intervals of storage. The NR activity at zero hr of storage was 0.98 units/g fr. tissue.

hr of storage in the spermine-treated tissue did not differ from that in the control. This suggests that spermine probably affects the synthesis of NR and not the breakdown. The fact that the effect of spermine was not observed during the storage of the homogenate also supports this possibility, since homogenization would cause disruption of organelles and protein synthesizing machinery.

Table 3. Effect of spermine and cycloheximide at different periods of induction of nitrate reductase in excised radish cotyledons

Compound added during induction at (hr)	Nitrate reductase activity % of control	
	+spermine	+cycloheximide
0	39	11
0.5	44	22
1.0	87	40
2.0	102	70
2.5	106	100

Cotyledons of 36 hr old dark grown seeds were excised and treated with spermine (1 mM) or cycloheximide (2 μg/ml) during induction as specified. Induction was carried out with KNO_3 (10 mM) for 3 hr. The enzyme activity in the control group was 0.93 units/g fr. tissue.

To determine whether spermine interferes with the induction of the enzyme, the effect of spermine and cycloheximide was studied when added at different intervals of induction (Table 3). Both spermine and cycloheximide were effective when added along with nitrate. Cycloheximide was more effective than spermine. The inhibitory effect of both compounds decreased when added at later stages of induction and there was no effect when spermine was added at the 2nd hr of induction while cycloheximide caused only 30% inhibition. Thus spermine probably interfered with the *de novo* synthesis of NR.

NR, in addition to reducing nitrate, possesses two partial activities, cytochrome *c* reductase and methyl viologen-NR [9]. Several compounds like molybdenum, tungsten and sulphydryl reagents have been reported to affect their synthesis and activities differentially [9, 10]. When the effect of spermine was studied on these partial activities, it was found to inhibit both methyl viologen-NR as well as cytochrome *c* reductase activities by *ca* 60% (data not shown) suggesting that the synthesis of the whole NR complex was inhibited. Polyamines have generally been associated with promotion of protein synthesis by stabilizing ribosomes [11] and nucleic acids [12] though in some recent reports they have also been implicated with the inhibition of protein synthesis [13, 14]. The effect of polyamines of NR synthesis in the present study appears to belong to the second category.

EXPERIMENTAL

Plant material. Radish seeds (*Raphanus sativus* L. var. Suttons, Scarlet Globe), obtained from a local dealer, were grown in the dark for 36 hr in the absence of a nitrogen source. Cotyledons were excised and pretreated with the test compounds for 30 min before induction. NR was induced with KNO_3 (10 mM) for 3 hr in dark, unless otherwise specified. The concn of nitrate (10 mM) and 3 hr period of induction were found to be optimal in preliminary studies. At the end of the induction period, cotyledons were washed, and a 10% (w/v) homogenate was prepared by grinding the tissue in a pestle and mortar using 10 mM NaPi buffer, pH 7.5 containing 5 mM cysteine and 10 mM EDTA.

Enzyme assay. NR activity was assayed according to the method of ref. [15]. The assay system contained 100 μmol NaPi buffer, pH 7.5; 10 μmol KNO_3, 0.1 μmol NADH and 0.2 ml of enzyme in a total vol. of 4 ml. The reaction was terminated after 30 min by the addition of 0.5 ml of 1 M $Zn(OAc)_2$. In the control tubes, nitrate was added after the termination of the reaction. Nitrite formed was estimated according to the method of ref. [16].

Methyl viologen NR activity was assayed using methyl viologen, 0.1 ml (25 mg/10 ml) reduced with sodium dithionite, 0.1 ml (17 mg/ml) as an electron donor instead of NADH. The reaction was terminated after 30 min and nitrite was estimated as described above.

Cytochrome *c* reductase activity was assayed according to ref. [9]. The assay system consisted of 100 μmol NaPi buffer, pH 7.5; 0.05 ml (2% in H_2O) cytochrome *c*, 0.1 μmol NADH and 0.2 ml of enzyme in a total vol. of 3 ml. The reduction of cytochrome *c* was measured by following the rate of increase in *A* at 550 nm for 3 min. NADH was omitted from the control tubes. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 μmol of product per hr.

Nitrate content was estimated according to the method of ref. [17] and the metabolic pool was measured as described in ref. [18].

Chemicals. The amines, putrescine, spermidine, spermine and cycloheximide were purchased from Sigma. Other chemicals of analytical grade were purchased locally.

Acknowledgement—This work was supported by a grant from the Indian Council of Agricultural Research, New Delhi.

REFERENCES

1. Ferrari, T. E. and Varner, J. E. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **65**, 729.
2. Afridi, M. M. R. K. and Hewitt, E. J. (1965) *J. Exp. Botany* **16**, 628.
3. Schrader, L. E., Ritenour, G. L., Eilrich, G. L. and Hageman, R. H. (1968) *Plant Physiol.* **43**, 930.
4. Srivastava, S. K., Kansara, M. S. and Mungre, S. M. (1986) *Plant Growth Regul.* **3**, 339.
5. Rao, K. P. and Rains, D. W. (1976) *Plant Physiol.* **57**, 55.
6. Jackson, W. A., Flesher, D. and Hageman, R. H. (1973) *Plant Physiol.* **51**, 120.
7. Naik, B. I. and Srivastava, S. K. (1978) *Phytochemistry* **17**, 1885.
8. Aslam, M., Oaks, A. and Huffaker, R. C. (1976) *Plant Physiol.* **58**, 588.
9. Wray, J. L. and Filner, P. (1970) *Biochem. J.* **119**, 715.
10. Hewitt, E. J., Hucklesby, D. P., Mann, A. F., Notton, B. A. and Rucklidge, G. J. (1979) in *Nitrogen Assimilation of Plants* (Hewitt, E. J. and Cutting, C. V., eds) p. 255. Academic Press, New York.
11. Hardy, S.J. S. and Turncock, G. (1971) *Nature* **229**, 17.
12. Ames, B. N. and Dubin, D. T. (1960) *J. Biol. Chem.* **235**, 769.
13. Apelbaum, A., Icekson, I., Burgoon, A. C. and Lieberman, M. (1982) *Plant Physiol.* **70**, 1221.
14. Auberger, P., Samson, M., Lecam, G. and Lecam, A. (1983) *J. Cell. Physiol.* **117**, 204.
15. Hageman, R. H. and Hucklesby, D. P. (1971) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, H. O., eds) Vol. 23, p. 491. Academic Press, London.
16. Evans, H. G. and Nason, A. (1953) *Plant Physiol.* **28**, 233.
17. Sloan, C. H. and Sublett, B. J. (1966) *Tobacco Sci.* **10**, 121.
18. Ferrari, T. E., Yoder, O. C. and Filner, P. (1973) *Plant Physiol.* **51**, 423.