Significance of nitrate reductase in nitrate assimilation in free-living *Rhizobium* cultures

M.H. Siddiqui[1], Anjali Mathur and S.N. Mathur[2]

Plant Physiology Research Laboratory, Department of Botany, University of Gorakhpur, Gorakhpur-273001 (India), 6 November 1981

Summary. An in situ method for measuring nitrate reductase (NR) in free-living *Rhizobium* cultures has been developed. Arginine, threonine, valine, glutamic acid, tyrosine and tryptophan were found to promote NR synthesis. In root nodules rhizobial nitrate reductase also converts nitrate into NH_4^+, thus it provides an alternative to the nitrogenase route for the formation of NH_4^+.

The physiological significance of nitrate reductase (EC 1.6.6.2) which catalyses the reduction of inorganic nitrate to nitrite has been extensively studied in many groups of organisms, including bacteria[3] and higher plants[4], but reports of its presence in free-living cultures of *Rhizobium* are few[5,6]. *Rhizobium,* which lives in symbiotic association in root nodules of leguminous plants, is characterized by its ability to reduce atmospheric nitrogen to ammonia, which by the glutamine synthetase/glutamate synthase or glutamate dehydrogenase pathways[7] enters the amino acid pool. NR provides an alternative route for nitrogen assimilation in root nodules. Hence, measurement of its activity in free-living cultures should be of great interest. Several methods have been adopted to measure different enzymes in in situ by permeabilization with organic solvent (toluene) in enteric bacteria[8] and in yeast[9]. The present paper describes a modified assay procedure for measurement of NR in free-living *Rhizobium* cultures and also the effect of different nitrogen sources on its synthesis and activity.

Materials and methods. The culture of *Rhizobium leguminosarum* obtained from the Division of Microbiology, Indian Agricultural Research Institute, New Delhi, was maintained on autoclaved yeast extract mannitol broth[10], containing in 1 l 0.5 g K_2HPO_4, 0.2 g $MgSO_4$, 0.1 g NaCl, 3.0 g $CaCO_3$, 10.0 g mannitol and 0.5 g extract. The pH of the medium was adjusted to 7.0 before autoclaving. The culture was grown under aerobic condition in sterilized 250 ml Erlenmeyer flasks shaken in an automatic shaker with 120 strokes per min at 30 °C.

For growth studies, a *Rhizobium* inoculum of the same density was added to 5 ml culture broth in sterilized culture tubes each time, and the density of the bacterial population was measured at 610 nm at 0, 24, 48, 96 and 120 h after inoculation. The effects of KNO_3 (substrate), sodium tungstate (inhibitor), and sodium molybate (modulator) on NR activity were also studied at different times in exponentially grown cultures.

Assay of nitrate reductase (NR): In situ assay: Rhizobial cells from an exponentially growing culture were harvested by centrifugation at 10,000 × g at 0 °C. The cells were washed and centrifuged again. The NR-activity in the collected cells was assayed by incubating them in 2 ml assay mixture which contained 0.2 M phosphate buffer (pH 7.4), 16 mM KNO_3, and 0.12% n-propanol. The incubation was done for 1 h at 30 °C in a constant temperature incubator and NO_2^- formed was determined[11]. The absorbance of the pink color was read at 540 nm.

In vitro assay: The cells were harvested by centrifugation, washed and crushed in 1 ml chilled phosphate buffer (pH 7.4) at 0 °C with mortar and pestle. The extract was centrifuged at 10,000 × g for 10 min at 0 °C. The clear supernatant was taken as enzyme source. The assay mixture contained in 2 ml 0.5 ml enzyme solution, 0.2 M phosphate buffer (pH 7.4), 16 nM KNO_3, and 0.1 mM NADH. The other details are as in the in situ assay. The NR-activity is expressed in nmoles $NO_2^- h^{-1}$ (100 mg cells)$^{-1}$.

Analytical methods. Total amino acids[12], nitrate content[13] and total nitrogen[14] were also estimated in rhizobial cells.

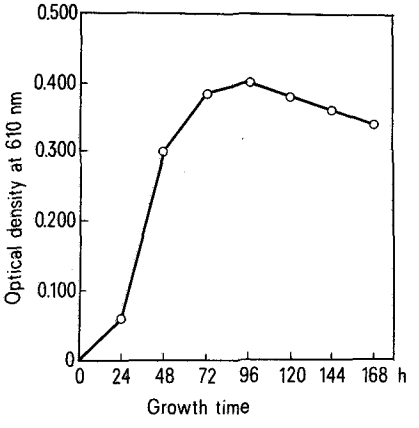

Figure 1. Bacterial growth in free-living *Rhizobium* culture at different times.

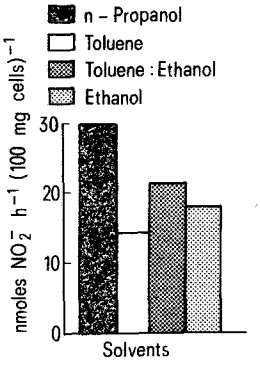

Figure 2. Effect of different solvents on nitrate reductase activity in free-living *Rhizobium.*

Table 1. Effect of culture conditions on different parameters in free-living *Rhizobium* cultures

Culture conditions	Nitrate reductase nmoles $NO_2^- h^{-1}$ (100 mg cells)$^{-1}$	Nitrate content mg N as NO_3^- (100 mg cells)$^{-1}$	Total free amino acid mg amino acid (100 mg cells)$^{-1}$	Total nitrogen μg N (100 mg cells)$^{-1}$
$+NO_3$	39.66 ± 0.33*	0.231 ± 0.006	0.227 ± 0.004	33.25 ± 0.09
$-NO_3$	21.33 ± 0.68	0.044 ± 0.006	0.131 ± 0.003	9.48 ± 0.19

* Mean ± SE (N = 3).

Table 2. Effect of amino acids on the synthesis of nitrate reductase. For this *Rhizobium* was grown in nitrogen-free YEMA medium with different amino acids at 0.2 mM concentration, until the exponential phase was reached. The control was obtained from the culture in nitrogen-free YEMA medium only

Amino acids	Nitrate reductase activity nmoles $NO_2^-h^{-1}$ (100 mg cells)$^{-1}$	% of control
Control	20.50±0.28*	100
Arginine	54.00±0.94	261.46
Threonine	52.00±0.55	253.65
Valine	76.66±0.07	373.95
Glutamic acid	32.00±1.04	156.09
Alanine	23.00±0.57	112.19
Tryptophan	27.33±0.59	133.31
Tyrosine	30.00±0.28	146.34
Leucine	9.00±0.28	44.86
Cysteine	7.00±0.28	34.14
Glutamine	14.50±0.28	70.73
Glycine	24.16±0.59	110.75
Asparagine	17.83±0.57	86.97
Aspartic acid	14.33±0.59	69.90

* Mean ± SE (N = 3).

All the experiments were done in triplicate and were repeated 3 times.

Results. Bacterial population. The *Rhizobium* was subcultured in YEMA medium and the growth, which was maximum at 96 h, was measured optically up to 168 h. No further increase in growth was observed after 96 h. The exponential growth phase lay between 24 and 48 h and is shown in figure 1.

Estimation of NR in cell-free extract and in in situ. Permeabilization of membranes with organic solvents has been used to assay various enzymes in in vivo[15,16]. Hence, several solvent systems were tried to increase cell permeability in the *Rhizobium* cells. However, 0.12% n-propanol was found to be most effective; other solvents such as toluene, ethanol, and toluene : ethanol (1 : 4) were less effective (fig. 2).

As shown in figure 3 the nitrate reductase activity was linear with time in cell-free extracts as well as with the in situ method using n-propanol; however, more activity was obtained in the latter.

Standardization of the method. To standardize any method (e.g. the in situ method described here, it is necessary to compare all the assay conditions with those of an accepted one (the in vitro method). Our results show that the same pH optima (7.4) and the same K_m for KNO_3 (1.38 mM) were found under both the assay conditions (fig. 4).

When the relation between NR, amino-acids, NO_3^- content and total nitrogen in culture grown in presence and absence of KNO_3 was measured, higher values were obtained in a NO_3^- grown culture than in a culture grown in its absence (table 1).

Inhibition study. When an exponentially-grown nitrogen-free culture was given different treatments for various periods it was found that sodium tungstate caused inhibition, whereas sodium molybdate intensified NR-activity more than did KNO_3; in the control, NR-activity remained almost constant throughout the experimental period (fig. 5). When these effectors were used in the culture medium during growth similar effects were observed, i.e. inhibiton by sodium tungstate and promotion of NR-activity by sodium molybdate.

Effect of different amino acids. Several amino acids (as given in table 2) were used in the culture medium. It was found that arginine, threonine, valine, glutamic acid, alanine, tryptophan, tyrosine, and glycine promoted NR-activity, while leucine, cysteine, glutamine, asparagine and aspartic acid were inhibory. Radin[17] made similar observations on the induction of NR-activity in cotton plants.

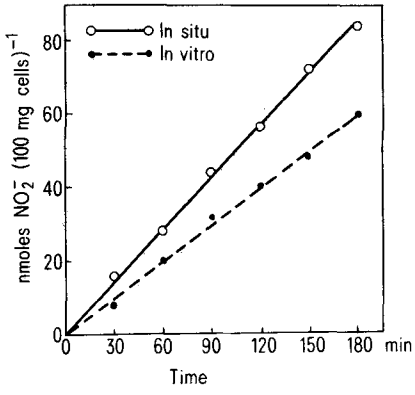

Figure 3. Time course of nitrate reductase in free-living *Rhizobium*, in situ assay (○——○), in vitro assay (●——●).

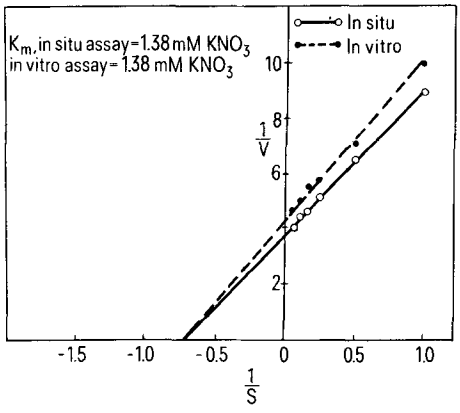

Figure 4. Lineweaver-Burk plot showing effect of different concentrations of KNO_3 on nitrate reductase activity in free-living *Rhizobium*; in situ assay (○——○), in vitro assay (●——●). The concentrations of KNO_3 are given in mM and NR-activity in nmoles $NO_2^-h^{-1}$ (100 mg cells)$^{-1}$.

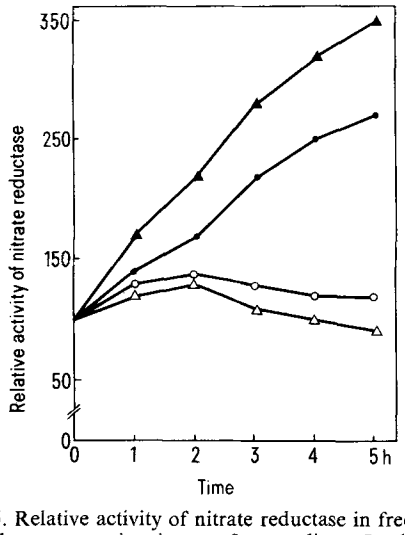

Figure 5. Relative activity of nitrate reductase in free-living *Rhizobium* culture grown in nitrogen-free medium. In the exponential phase the cells were harvested and given different treatments for different time; by water only (○——○), by 5 mM potassium nitrate (●——●), by 5 mM potassium nitrate + 10 µM sodium tungstate (△——△) and by 5 mM potassium nitrate + 10 µM sodium molybdate (▲——▲). Initial activity at 0 h is 20 nmoles $NO_2^-h^{-1}$ (100 mg cells)$^{-1}$.

Discussion. Assay of an enzyme's activity in situ provides a better understanding than assay in vitro, where the conditions may be altered during its extraction. For in vivo studies the permeability barrier causes serious difficulties. Permeabilization of membranes with organic solvents has been successfully done by several workers[15,18]. Choudary and Rao[9] used a mixture of toluene and ethanol to permeabilize yeast cells and studied NR-activity in situ. But in comparison to these solvents, n-propanol gave more NR-activity in free-living *Rhizobium* cultures. Studies with tungstate and molybdate further confirmed the in situ activity observed. Inhibitory effects of tungstate on NR-synthesis and on its catalysing capacity have been reported for *Chlorella*[19] and higher plants[20] respectively. These findings suggest that NR present in bacteria behaves in a way similar to that of higher plants. The results with amino acids showed that the de novo synthesis of NR in nitrogen-free medium was affected by them, as shown by others in different micro-organisms[21].

From the present observations it may be concluded that nitrate present in the soil can be reduced to NH_3 by rhizobial NR and nitrite reductase (NiR). Thus in legume root nodules, besides nitrogenase, NR in an additional enzyme that provides an alternative route for the formation of NH_4, as shown in free-living *Rhizobium* cultures in the present study.

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Acid phosphatase activity of small intestine of mice after exposure to different doses of gamma rays

K. Nandchahal, P. Uma Devi and P.N. Srivastava

Radiation Biology Laboratory, Department of Zoology, University of Rajasthan, Jaipur-302 004 (India), 9 September 1981

Summary. Effect of whole-body radiation at 3 different dose levels on the activity of acid phosphatase was studied in the small intestine of Swiss albino mice. In all the 3 exposure groups the enzyme activity increased significantly at 24 h after irradiation; the time at which the maximum histological damage was seen. With the beginning of recovery the enzyme tended to decrease and gradually approached control values.

Acid phosphatase is a hydrolytic enzyme localized in lysosomes[1]. Increase in acid hydrolases after irradiation seems to be characteristic of tissue damage by irradiation and has been reported for thymus[2], spleen[3], liver[4] and adrenal glands[5]. A similar post-irradiation increase in the acid phosphatase content of the small intestine has been demonstrated by histochemical studies[6-11]. In the present work an attempt has been made to study the post-irradiation changes in the activity of acid phosphatase in the small intestine of mice by biochemical methods.

Material and methods. Young adult male albino mice of 8-10 weeks weighing 24 g on the average were selected from an inbred colony for all the experiments. The animals were exposed to 450, 900 or 1200 R of whole body gamma radiation from a ^{60}Co source at the rate of 25 R/min and were sacrificed by cervical dislocation at 6 h, 1, 2, 4, 6, 8 and 14 days after irradiation. Exteriorized small intestine from duodenum to ileocoecal junction was immediately removed without the adhering mensenteric blood vessels and fat, slit longitudinally, rinsed thoroughly in ice cold 0.9% sodium chloride solution, minced into small fragments in a chilled petri-dish and homogenized in distilled water. The acid phosphatase activity in this homogenate was estimated by the method of Fiske and Subbarow[12]. Reading were taken on a Klett Summerson colorimeter and the activity of the enzyme was expressed as mg Pi/g/h. Statistical analysis was done using Student's t-test.

Results and discussion. Post-irradiation changes in the activity of the enzyme are presented in the histogram. After all the 3 doses an increase in the activity of the enzyme was evident during the early intervals. The peak values were obtained at 1 day after exposure when the activity in all the