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# Sources of reducing equivalents for nitrate reductase in *Pisum arvense* roots

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Abstract

The sources of the nitrate reductase (E.C 1.6.6.1) reducing equivalents were searched for in roots of *Pisum arvense* by measuring *in vivo* and *in vitro* nitrate reductase activity. It was found that the $NADH_2$ utilised in the process of nitrate reduction in the roots of *P. arvense* may be formed by glycolysis as well as in processes of organic acids oxidation such as 2-oxoglutaric, succinic and malic acids.

*Key words: nitrate reductase, reducing equivalents, Pisum arvense, roots*

## INTRODUCTION

The activity of nitrate reductase, the first enzyme on the nitrate reduction pathawy, is specifically dependent on $NADH_2$ as electron donor (Beevers and Hageman 1969, Hewitt 1975). According to the investigations of Klepper et al. (1971). $NADH_2$ utilised during nitrate reduction is produced in the process of glycolysis as the result of oxidation of triose phosphates. Lips (1971) claimed that one of main sources of nitrate reductase reductors in green tissues is the reaction catalysed by glycolate dehydrogenase. Similar suggestions were advanced by Plaut and Litton (1974). Neyra and Hageman (1978) demonstrated that $NADH_2$ for nitrate reduction in maize leaves (plants of type C-4) is probably formed by oxidation of malate produced during photosynthesis. Similarly Mann et al. (1978), and also earlier Mulder et al. (1959) ascertained that in spinach leaves (plants of type C-3) oxidation of malate or isocitrate may supply $NADH_2$ for nitrate

Abbreviations used: NR — nitrate reductase, MDH — malate dehydrogenase.

reduction. All the above quoted papers concerned green tissues. It is known, however, that nitrate reduction occurs as well in roots (Sanderson and Cocking 1964, Buczek 1976). $NADH_2$ utilised in the nitrate reduction is also produced in roots. However, apart from two communications of Deane-Drumonnd et al. (1979, 1980) suggesting the participation of malate dyhdrogenase in production of reduction power for NR activity in barley roots, data are lacking as regards the sources of reducing equivalents for the activity of this enzyme in roots. In view of this situation the present investigations were undertaken with the purpose of establishing the main metabolic pathways which might potentially lead to $NADH_2$ formation for nitrate reductase. As model plant *Pisum arvense* was chosen.

## MATERIAL AND METHODS

The experiments were performed on roots of *Pisum arvense* L. cultivated in hydroponic cultures. The seeds were germinated for three days in darkness at 27°C. Equal-sized seedlings were transferred to glass crystallisers, covered with aluminium foil and filled with preliminary nutrient medium deprived of nitrogen and containing the following components, in $\mu$moles: $K_2SO_4$—3.0, $CaH_2PO_4 \cdot H_2O$—1.0, $MgSO_4 \cdot 7H_2O$—1.0 and Fe-citrate — 0.017. One cu. cm of microelements was added to the medium (0.021 mg $H_3BO_3$, 0.241 mg $MnSO_4 \cdot 5H_2O$, 0.025 mg $CuSO_4 \cdot 5H_2O$, 0.025 mg $ZnSO_4 \cdot 7H_2O$, 0.0024 mg $CoSO_4 \cdot 7H_2O$, 0.012 mg $Na_2MoO_4 \cdot 2H_2O$) and the plants were placed in a glasshouse. After three days of growth the remains of seeds were removed and the seedlings were transferred to 500 $cm^3$ beakers filled with Hoagland's medium consisting of (mmoles $\cdot dm^{-3}$): $Ca(NO_3)_2$ — 5, $KNO_3$ — 5, $KH_2PO_4$ — 1 with microelements added as specified above. pH of both media was adjusted to 6.5.

**Nitrate reductase activity (in vitro).** Roots of 9-day *P. arvense* seedlings were used for analysis. Enzymic extracts were prepared in 0.05 M Tris-HCl buffer (pH 7.8) containing 5 mM cystein and 2.5 mM EDTA. NR activity was determined by the method of Hageman and Flesher (1960). The reaction mixture contained 100 $\mu$moles phosphate buffer, pH 7.5, 20$\mu$moles $KNO_3$, 0.7 $\mu$mole $NADH_2$ and 0.2 $cm^3$ enzymic extract. The total volume of the mixture was 2 $cm^3$. After incubating the samples for 30 min at 27°C the reaction was stopped by addition of 0.5 $cm^3$ of 30 mM zinc acetate solution. The precipitate was centrifuged off for 10 min at 20 000 × g and the amount of nitrites formed during the reaction was determined colorimetrically (Harper and Hageman 1972) in the supernatant.

**Nitrate reductase activity (in vivo)** was measured by the method described by Jaworski (1971). Roots of the 9-old day of *P. arvense*

were placed in Erlenmayer flasks containing 500 μmoles of K-phosphate buffer (pH 7.5), 10 μmoles $KNO_3$ and 1 per cent 1-propanol. Total volume of incubation medium was 5 $cm^3$. The samples were incubated for 1 hr in darkness, at 30° C and the amount of nitrite produced was determined (Harper and Hageman 1972).

## RESULTS

The data shown in Table 1 indicate that $NADPH_2$, in contrast to $NADH_2$, is not an effective electron donor for nitrate reductase in *P. arvense* roots. Addition of sugar phophates into the incubation medium distinctly stimulated NR *in vivo*, the increase of activity being highest in the presence of fructose-1,6-diphosphate. Organic acids such as malic, 2-oxoglutaric and succinic acid present in the incubation medium containing also $NAD^+$ or $FAD^+$ did not affect NR activity.

Table 1

Influence of various substrates (25 μM) added to the incubation medium on nitrate reductase activity *in vivo* in *Pisum arvense* roots

| Substrate | Nitrate reductase activity | |
|---|---|---|
| | nmole $NO_2 \cdot g^{-1}$ fr. wt. $\cdot h^{-1}$ | % |
| Control* | 758.1±23.4 | 100 |
| ATP | 745.5±21.8 | 98 |
| $NAD^+$ | 732.2±21.3 | 96 |
| $NADPH_2$ | 601.2±24.5 | 79 |
| $NADH_2$ | 951.1±34.2 | 125 |
| Glucose-6-phosphate+$NAD^+$+ATP | 1824.6±63.7 | 225 |
| Fructose-6-phosphate+$NAD^+$+ATP | 1814.6±60.8 | 239 |
| Fructose-1,6-diphosphate+$NAD^+$+ATP | 2113.4±69.1 | 279 |
| Malic acid+$NAD^+$ | 524.1±20.2 | 69 |
| 2-oxoglutaric acid+$NAD^+$ | 642.3±20.7 | 85 |
| Succinic acid+$FAD^+$ | 640.0±21.4 | 85 |

* The samples contained phosphate buffer, propanol and $KNO_3$ in concentrations given in Material and methods. The results are means of five replications. The differences between arithmetic means of two different combinations exceeding two times the SE value are considered as significant.

The influence of the tested organic acids on NR activity became pronounced if the *P. arvense* roots, before being placed in the incubation medium were preincubated for 2 h in a solution of these acids in the presence of $NAD^+$ or $FAD^+$ (Table 2). Root preincubation, however, in a buffer with organic acids but without $NAD^+$ or $FAD^+$ did not produce significant changes in NR activity. It was found at the same time that addition of malonic acid to the incubation medium inhibited completely

NR activity and depressed the stimulating effect of succinic acid and 2-oxoglutaric acid on the enzyme activity. In the presence of malonic acid NR activation by malic acid and $NAD^+$ was also weaker.

Table 2

*In vivo* activity of nitrate reductase of *Pisum arvense* roots preincubated for 2 h in 25 mM solution of organic acids

| Substrates | Nitrate reductase activity, nmoles $NO_2^- \cdot g^{-1}$ fr. wt. $\cdot h^{-1}$ |
|---|---|
| Control* | 266.40± 9.2 |
| Malonic acid | 18.60± 0.6 |
| Malic acid | 254.63±10.0 |
| Malic acid+$NAD^+$ | 780.55±23.1 |
| Malic acid+$NAD^+$+malonic acid | 495.05±17.6 |
| Succinic acid | 270.09± 8.3 |
| Succinic acid+$FAD^+$ | 602.06±21.8 |
| Succinic acid+$FAD^+$+malonic acid | 233.20± 7.9 |
| 2-oxoglutaric acid | 248.09± 8.3 |
| 2-oxoglutaric acid+$NAD^+$ | 959.04±27.4 |
| 2-oxoglutaric acid+$NAD^+$+malonic acid | 221.30± 9.1 |

* Control samples contained phosphate buffer, propanol and $KNO_3$ in concentrations mentioned in Material and methods. The roots cut from the plants were preincubated for 2 h in solutions of the substances mentioned in the table. After careful washing the roots were used to determination of nitrate reductase activity *in vivo* (see Material and methods). The results are means of five replications.

Changes in NR activity in *P. arvense* roots after preliminary 2-h treatment with fructose-1,6-diphosphate solution or malate in the presence of $NAD^+$ are shown in Table 3. The enzyme activity was determined by the *in vitro* method. In both cases a higher than in the control NR activity was noted, although the observed increase of NR activity was lower than that observed in stimulation *in vitro*.

Table 3

Influence of fructose-1,6-diphosphate and of malic acid on *in vitro* nitrate reductase activity of isolated from *Pisum arvense* roots

| Substrates | Nitrate reductase activity | |
|---|---|---|
| | nmole $NO_2^- \cdot g^{-1}$ fr. wt. $\cdot h^{-1}$ | % |
| Control | 37.3±1.2 | 100 |
| Fructose-1,6-diphosphate+$NAD^+$ | 55.9±1.8 | 150 |
| Malic acid+$NAD^+$ | 62.2±2.3 | 175 |

Roots cut from the plants were preincubated for 2 h in phosphate buffer, pH 7.5 containing $KNO_3$ (control samples) and 25 μmoles of the substrates mentioned in the Table. Then enzymic extracts were prepared from these roots and used to nitrate reductase activity determination *in vitro*. The results are means from five replications.

## DISCUSSION

The rise of the NR activity level in *P. arvense* roots incubated in the presence of glucose-6-phosphate, fructose-6-phosphate and fructose-1, 6-phosphate indicates that $NADH_2$ produced by glycolysis may be a suorce of reducing equivalents for NR. Stimulation of NR by glucose-6-phosphate seems to indicate that in nitrate reduction, $NADPH_2$ the reductor derived from the pentosephosphate pathawy also can be utilised. In the present experiments, however, the ineffectiveness of reduced nucleotide phosphate in the nitrate reduction has been demonstrated, this being manifested by a lack of NR activity stimulation in the presence of $NADPH_2$ (Table 1). Since cell membranes are but poorly permeable to pyridine nucleotides, the foregoing conclusions may arouse certain reservations. The fact, however, that, under identical conditions, addition of $NADH_2$ to the incubation medium as compared with that of the control (Table 1) enhanced NR activity suggests the possibility of penetration of pyridine nucleotides *in vivo* into the cell.

The high specificity of NR towards $NADH_2$ as electron donor is a proven fact (Hewitt 1975). Wells and Hageman (1974) actually reported the participation of $NADPH_2$ in the process of nitrate reduction, but only in plants containing phosphatases which catalyse the transmition of $NADPH_2$ to $NADH_2$ (spinach, some maize genotypes). In the light of the present results, however, it seems that NR of *P. arvense* roots is specific towards $NADH_2$ as electron donor.

Some metabolites of the Krebs cycle such as 2-oxoglutaric, succinic and malic acids enhanced markedly in *P. arvense* roots the NR activity level measured by the *in vivo* method. Their effectiveness was noticeable, however, only after 2-h preincubation of the roots in a solution of the above named acids, but only in the presence of $NAD^+$ or $FAD^+$. A similar result was obtained by measuring *in vitro* NR activity after preincubation of the roots in malic acid solution with $NAD^+$. Since preincubation of *P. arvense* roots in malic acid and $NAD^+$ solution raised the NR activity measured both *in vivo* and *in vitro*, it may be supposed that malate influences the enzymatic protein level. This, however, seems but little probable since treatment of roots with a solution of the organic acid alone did not cause changes in NR activity (Table 1). Mann et al. (1978) made similar observations. It is, therefore, more probable that the increased NR activity noted after incubation of roots in organic acid solutions with $NAD^+$ may be the result of changes in the amount of reducing equivalents utilised by the enzyme. Thus, enhancement of NR activity observed in the presence of malic acid might be explained by an increase in the cytoplasmic pool of $NADH_2$, owing to oxidation of malic acid by cytoplasmic malate dehydrogenase (Yamazaki and Tolbert 1969). Such a relation was found in maize leaves (Neyra

and Hageman 1978) and spinach (Rathman 1978, Mann et al. 1978, Woo and Canvin 1980). The participation of cytoplasmic malate dehydrogenase in supplying $NADH_2$ for the process of nitrate reduction has also been suggested by Deane-Drummond et al. (1979, 1980) in barley roots.

In the present experiments, however, stimulation of NR activity was observed also in the presence of 2-oxoglutarate and succinate, this suggesting a role of mitochondria of root cells in the supply of reducing equivalents for the process of nitrate reduction. This supposition seems the more probable since malonic acid — a competitive inhibitor of succinate dehydrogenase, one of the enzymes of the Krebs cycle — neutralised the stimulating action of 2-oxoglutaric and succinic acids on NR activity. On the other hand, NR activation noted after preincubation of *P. arvense* roots in malic acid solution was only partly abolished by addition of malonic acid. This fact seems to confirm our supposition that both cytoplasmic and mitrochondrial malate dehydrogenase are involved in supplying reducing power of NR activity.

The action of all the tested organic acids in the present experiments on NR was retarded as compared with that of sugars and appeared as late as after 2-h preincubation of roots in acid solution. This delay may have been due to the different intracellular localisation of the processes of $NADH_2$ production and $NADH_2$-dependent nitrate reduction.

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## *Źródła równoważników redukcyjnych redukatazy azotanowej w korzeniach Pisum arvense*

Streszczenie

W pracy podjęto badania nad źródłem reduktora reduktazy azotanowej (E.C 1.6.6.1) w korzeniach *Pisum arvense* stosując pomiary *in vivo* i *in vitro* aktywności NR. Stwierdzono, że $NADH_2$ wykorzystywany w procesie redukcji azotanów w korzeniach peluszki może pochodzić zarówno z przemian glikolitycznych, jak również z procesów utleniania kwasów organicznych, takich jak kwas α-ketoglutarowy, bursztynowy i jabłkowy.