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# A CONSTITUTIVE COMPONENT OF NITRATE REDUCTASE IN BARLEY LEAVES

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ABSTRACT

Nitrite activates a constitutive component of nitrate reductase in barley plants under conditions in which the component is found free and not incorporated into the complete enzyme. The constitutive component is found free both in plants without nitrate and when nitrate reductase is inactivated with cyanide. The inhibition of nitrate reductase by cyanide seemed to initiate the loosening or separation of the component parts of the enzyme.

Nitrate reductase (EC 1.6.6.2) is a substrate-inducible enzyme (Hewitt, 1975). In some cases, however, nitrite elicited nitrate reductase activity (Ingle et al., 1966), but further investigation of the phenomenon was not pursued. Later, after experiments on the apparent induction of nitrate reductase (NR) by nitrite in bean seeds (Lips et al., 1973), we reported on the rapid appearance of nitrate reductase activity in nitrate-depleted leaves following their incubation in nitrite (Kaplan et al., 1974a). We suggested then that NR may be a product-inducible enzyme (Kaplan et al., 1974b). However, we found later that addition of nitrite to nitrate-free homogenates of barley leaves, even at low temperatures, elicited immediate NR activity (Kaplan et al., 1978). This observation led us to consider that nitrite could be an activator of nitrate reductase rather than an inducer. The possible existence of two forms of nitrate reductase, one active and another inactive was supported by the finding of such forms in *Chlorella* by Solomonson et al. (1975). In the case of *Chlorella* the inactive form was formed after treating NR with cyanide in the presence of NADH. This inactive form of NR was reactivated by ferricyanide or other oxidants, while nitrite protected the enzyme from inactivation by cyanide (Solomonson, 1974). Reactivation of cyanide-inhibited NR by ferricyanide was also reported in spinach leaves (Palacian et al., 1974) and maize (Wallace, 1975).

In this work we report on the effect of cyanide and nitrite on nitrate reductase activity in homogenates of barley leaves.

*Abbreviations used in text:* NR – nitrate reductase, NADH – reduced nicotinamide adenine dinucleotide, NR–$NO_2^-$ – nitrite-activated nitrate reductase, NR–$NO_3^-$ –

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nitrate-induced nitrate reductase, EDTA – ethylenediaminetetraacetate, FAD – flavine adenine dinucleotide.

## MATERIALS AND METHODS

*Plant material*. Barley (*Hordeum vulgare* L. cv. Dvir) leaves were obtained from seedlings 7–9 days old, which had been grown on 0.5 mM $CaSO_4$ or 50% Hoagland solution (Hoagland and Arnon, 1950).

*Homogenization*. Leaves were ground in 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA, using an ice-cold mortar and pestle. Two ml medium were used per gram fresh weight of leaves. The homogenate was filtered through 2 layers of gauze and the filtrate centrifuged at 20,000*g* for 15 min. The pellet was discarded and the supernatant used as the enzyme extract.

*Activation*. $KNO_2$ was added to the enzyme extract to a final concentration of 0.5 mM and the mixture was incubated at 30°C for 5 min. The resulting nitrate reductase activity enhanced by $NO_2^-$ was designated NR–$NO_2^-$ activity to distinguish it from the activity of the enzyme induced in plants grown in solutions containing nitrate, which was called NR–$NO_3^-$ activity.

*NR inactivation by cyanide*. Nitrate-induced NR (NR–$NO_3^-$) was inactivated by adding KCN, up to the concentrations indicated in each case, in the presence of 0.25 mM NADH.

*Nitrate reductase*. Enzyme activity was determined essentially as described by Hewitt and Nicholas (1964). Assays were performed in test tubes in a water bath at 30°C. Each tube contained 2 ml assay mixture, consisting of 0.2 ml of 0.1 M $KNO_3$; 0.2 ml of $2.6 \times 10^{-5}$M FAD; 0.4 ml of 2 mg/ml NADH; 0.1 ml of 1 M phosphate buffer, pH 7.4; 0.1 ml of 1% Triton x-100; 0.1 ml extract and 0.9 ml water. Nitrite was determined at 0 time (before starting the incubation at 30°C) and 15 min after the start of the reaction. Enzyme activity is expressed as the increase in nitrite, in µmoles per gram fresh weight of leaves per hour.

*NADH cytochrome c reductase*. Enzyme activity was determined essentially as described by Wray and Filner (1970). The assay mixture consists of 0.15 ml of 1 M phosphate buffer, pH 7.4; 0.15 ml of 2% cytochrome *c*; 0.3 ml of 2 mg/ml NADH; 0.05–0.1 ml extract and water was added to a final volume of 3 ml. The increase in the absorbance at 550nm as a result of the reaction of cytochrome *c* in the presence of NADH against a control lacking NADH was followed. An extinction coefficient of $\epsilon_{550} = 21\ mM^{-1}cm^{-1}$ was used to calculate the amount of cytochrome *c* reduced. Enzyme activity is expressed as µmoles of cytochrome *c* reduced per gram fresh weight of leaves per hour.

*Protein*. Protein was estimated in two ways: (a) the Lowry method (Lowry et al., 1951), and (b) absorbance at 230 and 260nm. Protein concentration was calculated according to the equation:

(protein) $\mu g = 183 \times \text{abs } 230\text{nm} - 75.8 \times \text{abs } 260\text{nm}$ (Kalb & Bernlohr, 1977).

*Chromatography*. The 20,000*g* supernatant obtained from a leaf homogenate was passed through a Sepharose Cl-6B column (43 × 3 cm), previously equilibrated with 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA. Protein was eluted from the column with the same buffer at a rate of 1.2 ml/min.

The fraction containing NR and the associated NADH cytochrome *c* reductase was collected from the Sepharose column and added to a Sephadex G-25 column (25 × 2 cm) before or after treatment with cyanide. The Sephadex G-25 column was previously equilibrated with 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA. Protein was eluted using the same buffer at a rate of 1.6 ml/min. Using this technique low molecular weight material was removed by fractionation of the extract on the Sepharose column so that only high molecular weight NR was loaded onto the Sephadex column. Therefore any low molecular weight material appearing after elution from the Sephadex column must have resulted from the treatment of the enzyme with $CN^-$ followed by $NO_2^-$.

## RESULTS

The largest activation of NR by nitrite was observed in homogenates of plants grown without nitrate. Nitrite itself had no effect on the activity of NR in homogenates of plants grown in Hoagland solution which had a high level of NR activity (Fig. 1). Gradual depletion of nitrate from plants grown in Hoagland solution, obtained by transferring the seedlings to 0.5 mM $CaSO_4$ solution, caused a decline of NR–$NO_3^-$ activity accompanied by an increase in NR–$NO_2^-$ activity (Fig. 1B). A reverse pattern was observed when plants were transferred from $CaSO_4$ to Hoagland solution (Fig. 1A). There seemed to be an inverse relationship between the activities of nitrate-induced (NR–$NO_3^-$) and nitrite-activated (NR–$NO_2^-$) forms of NR.

Cyanide, an NR inhibitor in higher plants (Hewitt, 1975) and algae (Solomonson, 1974), inhibited NR in homogenates of barley leaves (Fig. 2A), but did not affect NADH cytochrome *c* reductase associated with nitrate reductase. Nitrite protected the enzyme from inhibition by cyanide (Fig. 2B). Cyanide, when added to homogenates in the presence of nitrite, did not inhibit NR activity (Table I). However, cyanide-inhibited NR was partially reactivated by nitrite (Table II).

Nitrate reductase from various sources has been shown to bind cyanide (Solomonson, 1974; Hewitt, 1975), but neither ferricyanide nor nitrite caused release of radioactive $^{14}C$ supplied as $K^{14}CN$ from inactive NR of barley (unpubl. data).

Nitrate-induced NR was partially purified by eluting the 20,000*g* supernatant of a homogenate of plants grown on Hoagland medium from a Sepharose Cl-6B column (see Materials and Methods). Elution fractions containing NR and NADH cytochrome *c* reductase activities were collected and assayed (Fig. 3). When a partially purified

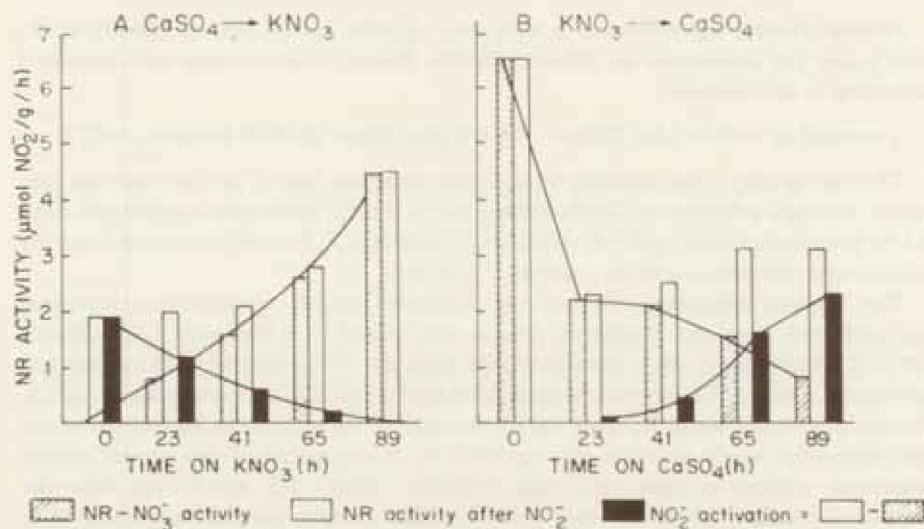


Fig. 1. NR activity in extracts of barley leaves as affected by nitrate during seedling growth. A. Seedlings grown in 0.5 mM $CaSO_4$ were transferred to 50% Hoagland solution (containing nitrate). B. Seedlings grown in 50% Hoagland solution were transferred to 0.5 mM $CaSO_4$ solution. At indicated time intervals plants were homogenized and nitrate reductase activity determined before and after activation with 0.5 mM $KNO_2$ for 5 min.

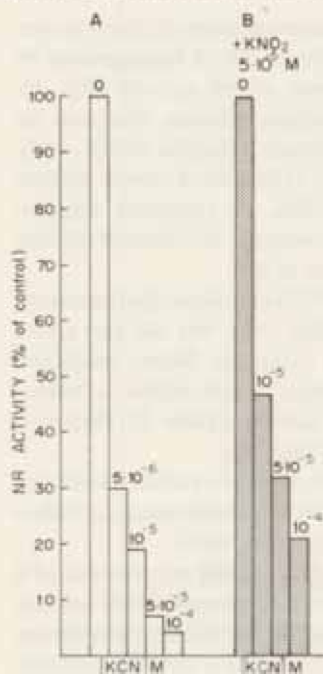


Fig. 2. Effect of nitrite on the in vitro reactivation of $CN^-$-inhibited NR in extracts of barley leaves. A. Inactivation of NR by various concentrations of cyanide. B. Effect of $5 \times 10^{-2}$ M $NO_2$ on the $CN^-$-inhibited NR.

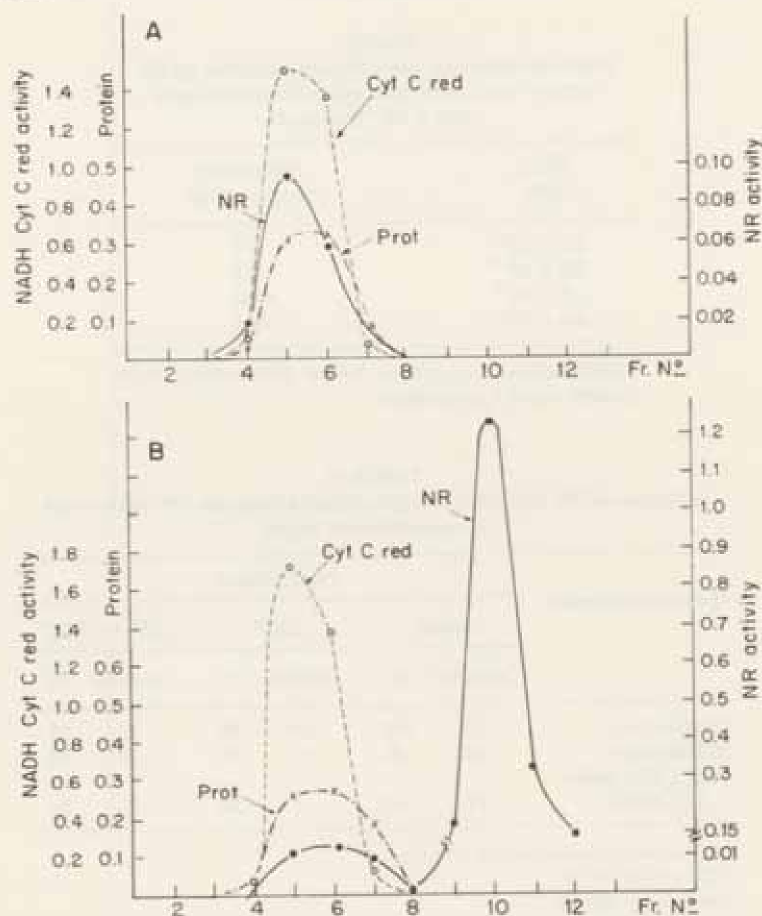


Fig. 3. Partial purification of active NR, obtained from barley leaves, on a Sepharose Cl-6B column. A. Nitrate reductase partially purified on Sepharose Cl-6B was loaded and eluted from a column of Sephadex G-25 without further treatment. B. After Sepharose, NR was pretreated with $5 \times 10^{-4}$ M KCN for 5 min followed by $5 \times 10^{-3}$ M $KNO_2$ for 5 min, then passed through Sephadex G-25. Enzyme activity expressed as μmol/ml/h. Protein concentration given as mg/ml.

preparation was passed through a Sephadex G-25 column without any pretreatment, NR and cytochrome *c* reductase activities were eluted in the void column (Fig. 3A). When the preparation was pretreated with cyanide, NR activity was inhibited and the elution profile was essentially the same, except that NR activity was very low. When pretreatment with cyanide was followed by addition of nitrite before transferring the extract through a Sephadex G-25 column an additional peak of NR activity appeared, while the cytochrome *c* reductase position was unaffected (Fig. 3B).

TABLE I
Effect of increasing concentrations of nitrite on NR activity in extracts from barley leaves pretreated with $5 \cdot 10^{-5}$ M cyanide

| *$KNO_2$ (M)* | *NR activity (% of control)*[1] |
|---|---|
| $2.5 \times 10^{-5}$ | 3.5 |
| $5.0 \times 10^{-5}$ | 8.5 |
| $1.0 \times 10^{-4}$ | 45.0 |
| $2.0 \times 10^{-4}$ | 92.0 |

[1] 100% activity in control before pretreatment with cyanide was 4.1 μmoles/g/h.

TABLE II
Inhibition of NR activity in extracts of barley leaves by $CN^-$ in vitro and its reactivation by nitrite

| *Fractions of extract* | *Pretreatment* | | | | | |
|---|---|---|---|---|---|---|
| | *None* | | *$CN^-$*[2] | | *$CN^- \rightarrow NO_2^-$*[3] | |
| | *Activity*[1] | *%* | *Activity* | *%* | *Activity* | *%* |
| Crude extract | 35 | 100 | 17 | 48 | 22 | 63 |
| 20000g super | 29 | 83 | 12 | 34 | 19 | 54 |
| $(NH_4)_2SO_4$ pellet (20–45%) | 15 | 43 | 7 | 20 | 11 | 31 |

[1] NR activity = μmol $NO_2^-$/g fr. wt/h.
[2] $CN^- = 5 \times 10^{-4}$ M.
[3] $NO_2^- = 5 \times 10^{-4}$ M.

Furthermore, no ninhydrin reactive products (amino acids) were found after acid hydrolysis (absorbance at 570nm) in the Sephadex G-25 eluate of an untreated, partially purified preparation of NR (Fig. 4A). Ninhydrin reactive compounds were found in the eluate of a cyanide-pretreated preparation (Fig. 4B). The position of the hydrolysed protein corresponded to that of NR–$NO_2^-$ (Fig. 3B).

Since low molecular weight compounds were removed from the preparation by passing the homogenate through a Sepharose Cl-6B column prior to any other step described above, the low molecular weight component found in the eluate of the Sephadex G-25 column resulted from the treatment of NR protein by cyanide and nitrite. The compound resulting from cyanide treatment and showing NR activity on addition of nitrite may be a low molecular weight (less than 5000) peptide (Figs. 3 & 4).

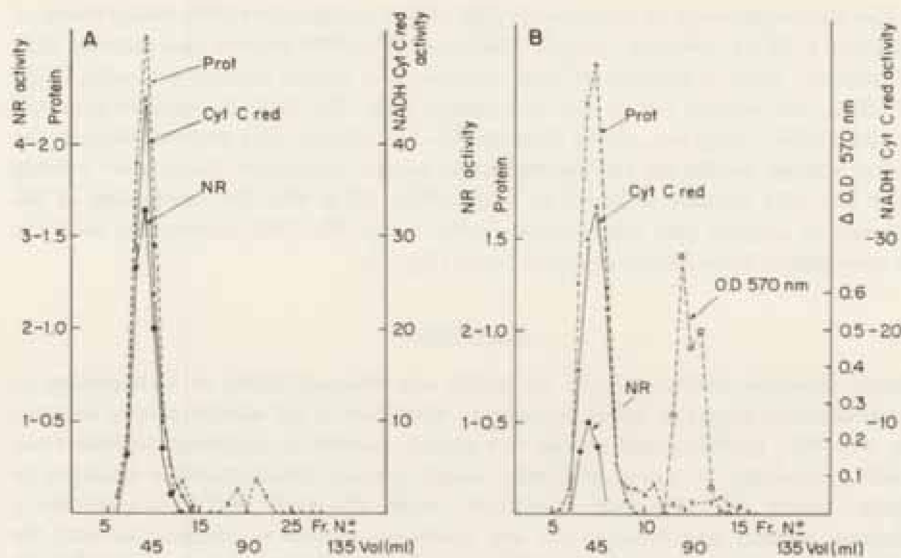


Fig. 4. Effect of cyanide on the activity of partially purified NR. A. Elution from Sephadex G-25 without further treatment. B. Elution from Sephadex G-25 after addition of 0.1 mM KCN to extract. Samples of each elution fraction were hydrolysed in 6M HCl and treated with ninhydrin. Absorbance at 570nm was measured before and after hydrolysis. Enzyme activity expressed as μmol/fraction/h and protein as mg/fraction.

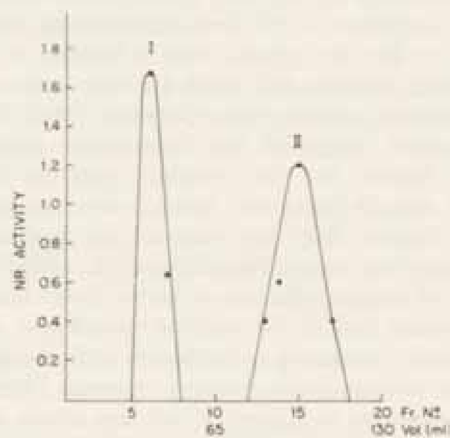


Fig. 5. Elution of NR from a Sephadex G-25 column. Homogenate of leaves from plants grown on 0.5 mM $CaSO_4$ and activated by nitrite was mixed with a homogenate from leaves of Hoagland-grown plants. I. NR activity of plants grown on Hoagland solutions (NR – $NO_3^-$). II. NR activity of homogenate of nitrate-deficient plants activated by nitrite (NR – $NO_2^-$). Enzyme activity expressed as μmoles of $NO_2^-$/ml eluent/h.

The elution patterns of the induced (NR–$NO_3^-$) and activated (NR–$NO_2^-$) forms of NR from a DEAE cellulose column (Kaplan et al., 1978) showed two proteins with NR activity. When a mixture of both enzymes was passed through Sephadex G-25, NR–$NO_3^-$ was washed out in the void volume (Fig. 5I), while the enzyme activated by nitrite (NR–$NO_2^-$) was eluted between 80–110 ml (Fig. 5II), corresponding to the elution volume needed for the low molecular weight component found after treating active NR with cyanide followed by nitrite (Figs. 3B & 4B). The component of NR detached by cyanide may therefore be similar to the NR–$NO_2^-$ activated by nitrite in the homogenate from nitrate-depleted plants (Fig. 5).

## DISCUSSION

Nitrate reductase activity elicited by nitrite was observed either in homogenates of nitrate-depleted plants or following cyanide inhibition of the nitrate-induced enzyme. The NR–$NO_2^-$ activity could be due to a moiety capable of accepting electrons from NADH, consisting of a low molecular weight peptide which transfers electrons to nitrate. Under NR induction conditions, when the large NADH cytochrome *c* reductase subunit was formed, the low molecular moiety is incorporated into the entire nitrate reductase enzyme, resulting in a diminished response to nitrite (Fig. 1).

Data presented in this paper and in a previous report (Kaplan et al., 1978) point to the possibility that nitrite activates a constitutive subunit of the NR protein, which may be the molybdenum subunit, while nitrate induces the dehydrogenase part of the protein which has the NADH cytochrome *c* reductase associated with NR.

The nitrite-activated subunit may be similar to the molybdenum-containing complex (MCC component) of NR from spinach leaves described by Hewitt et al. (1979), and the cnx component of NR from *Aspergillus* sp. described by Cove (1979). It might be similar to the Mo cofactor that is believed to be common in all the molybdenum-containing enzymes and which has been shown to contain or be associated with a low molecular weight protein (Johnson, 1980). The work of Nason et al. (1971) with *Neurospora* suggested the constitutive nature of the Mo-protein component of NR. Hewitt and his coworkers observed the splitting of nitrate reductase protein of spinach leaves into subunits when passing the enzyme through an AMP-Sepharose column. They also observed the appearance of a Mo–peptide component of low molecular weight (Rucklidge et al., 1976). We found that cyanide causes the splitting of nitrate reductase of barley leaves into two subunits: a high molecular weight protein capable of reducing cytochrome *c* and a low molecular weight peptide, perhaps containing molybdenum and associated with a peptide, as was found for other molybdenum-containing enzymes (Johnson, 1980). This low molecular weight component is capable of reducing nitrate upon pretreatment with nitrite. The effect of cyanide described here differs therefore from that found by Solomonson et al. (1973) for NR of *Chlorella*. In *Chlorella*, cyanide binds to NR protein and thus inhibits NR activity. However, enzyme activity is completely restored on addition of ferricyanide or other oxidants (Solomonson et al., 1973; Lorimer et al., 1974). It also differs from the situation described for spinach (Palacian et al., 1974)

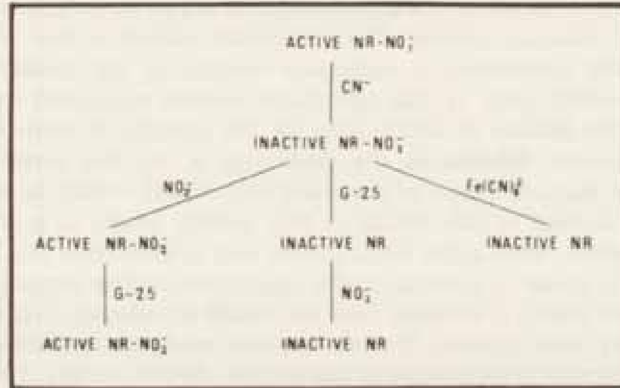


Fig. 6. Effects of cyanide and nitrite on nitrate reductase activity.

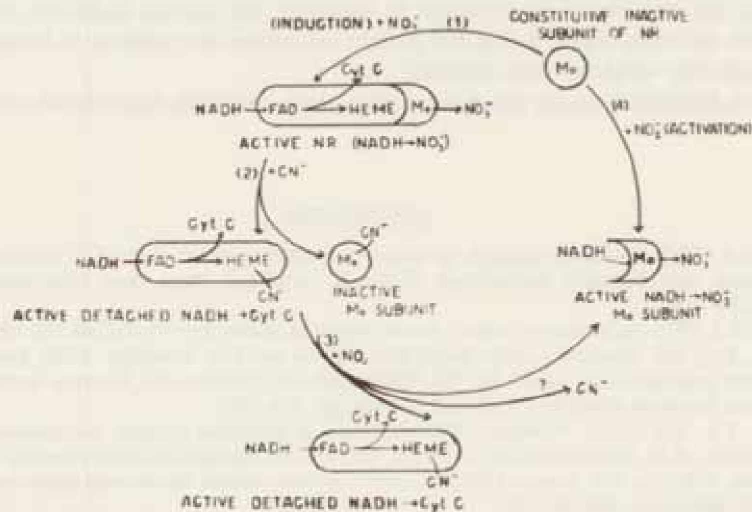


Fig. 7. A hypothetical model describing the relationships between inducible NR and the constitutive inactive subunit of NR.

and maize (Wallace, 1975), apparently because the conditions under which cyanide inhibited NR were different from the ones used in the present work. The effects of nitrite and cyanide on NR activity as observed in this work are summarized in Figure 6.

A hypothetical scheme summarizing the observations related to nitrate reductase in higher plants is presented in Figure 7. Barley and other plant species tested (Kaplan et al., 1978) contain a constitutive inactive component of nitrate reductase, very similar in its molecular weight to the one found in *Neurospora* (Nason et al., 1971), spinach (Hewitt et al., 1979) and in other molybdenum-containing enzymes (Johnson,

1980). Addition of nitrate to the nutrient medium caused the induction of the NADH cytochrome *c* reductase subunit. The constitutive subunit is then attached to the induced NADH cytochrome *c* reductase, resulting in the formation of active NR–NADH → $NO_3^-$ (step 1). The constitutive, inactive component can be activated by nitrite in the absence of nitrate (step 4). The capacity of nitrite to activate the inactive component depends on the availability of the free constitutive inactive subunit which becomes a part of the active NR–NADH → $NO_3^-$ in the presence of nitrate. The increase in NR–NADH → $NO_3^-$ activity results in a decrease of the response to nitrite. When active NR is treated with cyanide (step 2) a low molecular weight subunit, similar or identical to the constitutive inactive component present in nitrate-depleted plants, is detached from the NADH cytochrome *c* reductase subunit. The weakening bond between the two subunits results in formation of an active NADH cytochrome *c* reductase and an inactive NR–NADH → $NO_3^-$. Thus the cyanide treatment apparently increases the availability of a free constitutive component and the response to nitrite increases accordingly (step 3). The suggested process could also explain the inability of ferricyanide to reactivate the enzyme under our conditions. Cyanide not only binds to the NR protein but causes the splitting or loosening of a low molecular weight protein complex.

Work is presently under progress to characterize the small, constitutive, subunit.

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