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Effect of Cyanide and Nitrite on the Activity of Nitrate Reductase

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Nitrate reductase is generally considered to be a substrate-inducible enzyme (Hewitt, 1975). Nitrite, however, elicits nitrate reductase activity (Ingle *et al.*, 1966; Lips *et al.*, 1973) and led us initially to consider that NR could be a product-inducible enzyme (Kaplan *et al.*, 1974). Later studies in our laboratory indicated that nitrite added to homogenates of nitrate-depleted plants causes activation of nitrate reductase activity after a short time at low temperature (Kaplan *et al.*, 1978). We also reported that nitrate-induced NR ($NR\text{-}NO_3^-$) and nitrite-activated NR ($NR\text{-}NO_2^-$) are two distinct physical entities which can be separated on DEAE-cellulose columns (Kaplan *et al.*, 1978).

Leaves were obtained from barley (*Hordeum vulgare*, L. cv Dvir) seedlings grown in 0.5 mM $CaSO_4$ or 50% Hoagland solutions. Homogenization, activation and nitrate reductase assay were done as described previously (Kaplan *et al.*,

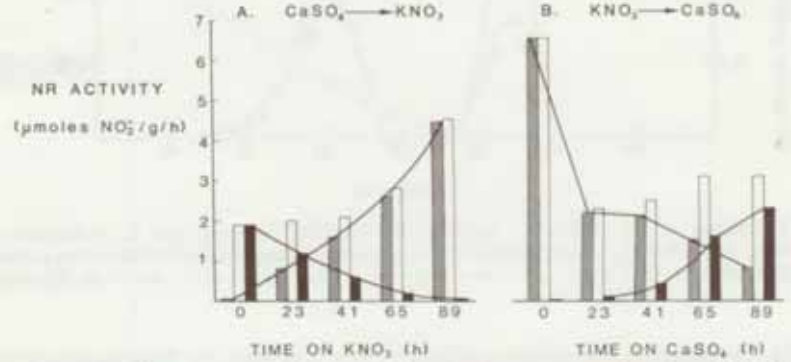

Fig. 1. A. Seedlings grown on 0.5 mM $CaSO_4$ solutions were transferred to 50% Hoagland solutions (containing NO_3^-). B. Seedlings grown on 50% Hoagland solutions were transferred to 0.5 mM $CaSO_4$ solutions. After periods of time indicated some of the plants were homogenized and NR activity determined before and after a 5 min treatment with 0.5 mM KNO_3. ■ $NR\text{-}NO_3^-$; □ $NR\text{-}NO_3^-$ after NO_3^-; ■ NO_3^- activation = □-■.

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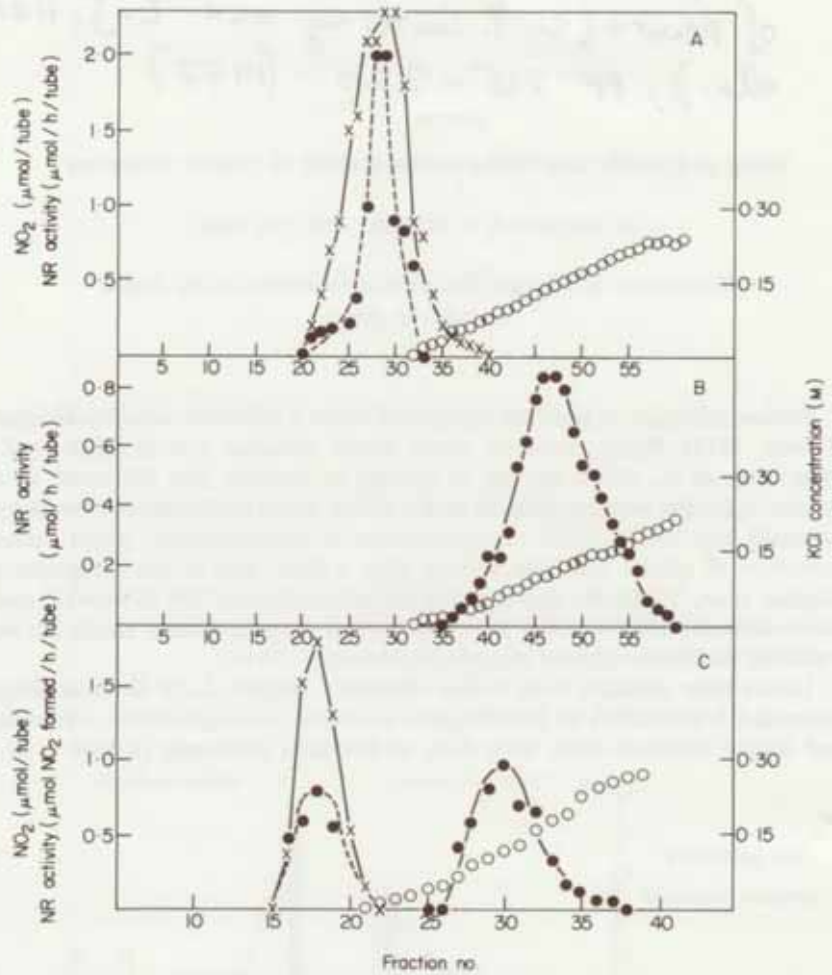

Fig. 2. Elution of: A, NR-NO_2^- activity; B, NR-NO_3^- activity; and C, a mixture of NR-NO_2^- and NR-NO_3^- activities from three parallel columns of DEAE-cellulose by a linear gradient of KCl. NR-NO_2^- ×——×——×——×; NO_2^- ●- - -●- - -●- - -●; NR-NO_3^- ●———●———●———●; KCl ○○○○.

1978). Cytochrome *c* reductase was assayed as described by Solomonson *et al.* (1973) and protein was estimated according to Lowry *et al.* (1951).

Maximal response to nitrite activation is found in nitrate-depleted plants, and no response at all is observed in homogenates obtained from plants grown in Hoagland solutions having substantial amounts of induced nitrate reductase

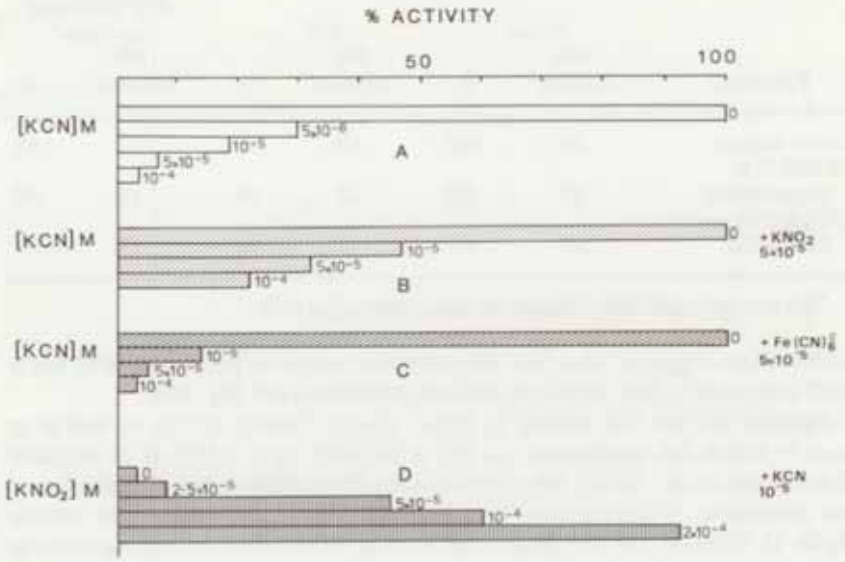

Fig. 3. Effect of NO_2^- and $Fe(CN)_6^{-3}$ on the *in vitro* inactivation of NR by CN^-. A. Inactivation of NR activity by various concentration of CN^-. B. Effect of the presence of 5×10^{-5} M NO_2^- in the reaction mixture, on the inactivation by CN^-. C. Effect of the presence of 5×10^{-5} M $Fe(CN)_6^{-3}$ in the reaction mixture on the inactivation by CN^-. D. Effect of various NO_2^- concentration on the inactivation by a constant concentration of CN^-.

(Fig. 1). A gradual depletion of nitrate from the plants, obtained by transferring the seedlings to 0.5 mM $CaSO_4$ solutions, shows that the decline in the amount of NR-NO_3^- is accompanied by a corresponding increase of NR-NO_2^-. In other words, there seems to be a close inverse relationship between the induced and activated forms of the enzyme suggesting that we may be dealing with two interchangeable forms of nitrate reductase.

The patterns from DEAE-cellulose columns (Fig. 2) indicate that the induced and the activated NR are different. The elution of NR-NO_2^- from a Sephadex

TABLE I

In vitro inhibition of NR activity by CN^- and reactivation by NO_2^-.

Fractions	Pretreatment					
	None		CN^-		CN^- followed by NO_2^-	
	NR activity[a]	%	NR activity[a]	%	NR activity[a]	%
Crude extract	35	100	17	48	22	63
20 000 × g supernatant	29	83	12	34	19	54
$(NH_4)_2SO_4$ pellet (20–45%)	15	45	7	20	11	31

[a] NR activity = μmol NO_2^- formed per gram fresh wt per hour.

G-25 column suggested also that the molecular weight of nitrite-activated NR is small compared to that of the nitrate-induced enzyme (cf. Fig. 4D).

Cyanide inhibits NR activity in higher plants (Hewitt, 1975), as well as in algae in which full reactivation of NR is obtained upon addition of oxidants (Solomonson *et al.*, 1973). We observed that CN^--inhibited NR of barley leaves was insensitive to ferricyanide but could be partially reactivated by nitrate (Table I). Cyanide did not inhibit NR activity when added to homogenates in the presence of nitrite at sufficient concentrations (Fig. 3).

Cyanide labelled as $^{14}CN^-$ is not released from the enzyme by ferricyanide or by nitrite even though the latter reactivates the enzyme. Nitrite seems to activate a low molecular weight component of NR which is split from the active enzyme by treatment with CN^- (Fig. 4). This component seems to be identical to the NR-NO_2^- activated by nitrite in nitrate-depleted plants.

In plant homogenates NR-NO_2^- shows up as a consequence either of nitrate depletion in the plants, or of cyanide inhibition. The effect of nitrite seems to be its ability to make the small NR component reactive with NADH, thus allowing it to reduce nitrate without intervention of the cytochrome *c* reductase component. The low molecular weight component seems to be very similar to the MCC cofactor described by Hewitt and co-workers (see this Volume, pp. 227–254 and 255–287), and to the *cnx* component of the *Aspergillus nidulans* enzyme described by Cove (see this Volume, pp. 289–297).

The results given here may constitute the missing link between the studies of Nason *et al.* (1971) with *Neurospora crassa* indicating the constitutive nature of the Mo-sub-unit of nitrate reductase, the work of the Long Ashton group with the MCC component of nitrate reductase which is split from the enzyme when

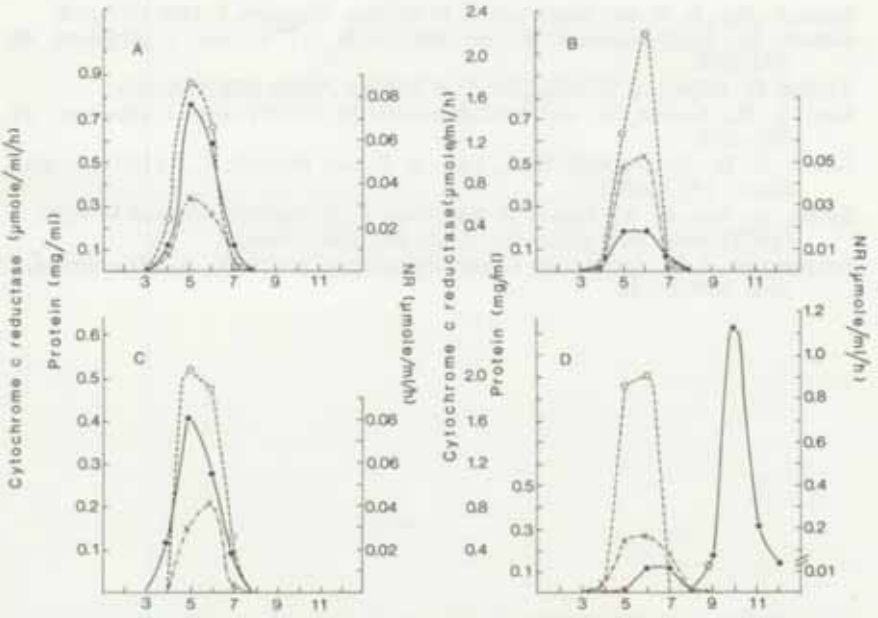

Fig. 4. Active NR obtained from plants grown on 50% Hoagland solutions and eluted from a sepharose 6B column. A. The active enzyme was put on a Sephadex G-25 column without any pretreatment. B. The active enzyme was pretreated with 5×10^{-4} M KCN and then put on a Sephadex G-25 column. C. The active enzyme was pretreated with 5×10^{-3} M KNO_3 and then put on a Sephadex G-25 column. D. The active enzyme was pretreated with 5×10^{-4} M KCN followed by treatment with 5×10^{-3} M KNO_3 and then put on a Sephadex G-25 column. NADH-NR ———; NADH cytochrome *c* reductase ○ — — ○; Protein × – · – · – ×.

transferring it through AMP-sepharose (Hewitt *et al.*, see this Volume, pp. 255–287) and the characteristics of cyanide inhibition of nitrate reductase in *Chlorella vulgaris* as described by Solomonson *et al.* (1973). It seems to us that the nitrite-activated component in nitrate-depleted plants is a constitutive sub-unit which becomes incorporated to the NR enzyme during the induced synthesis of the cytochrome *c* component.

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