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Control of Nitrate Reductase Activity in Barley Aleurone Layers*

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Abstract. Nitrate reductase activity in barley (*Hordeum vulgare* L. cv. Himalaya) aleurone layers has been determined in the intact tissue, using two different methods. The first method measures the rate of appearance of $H_2{}^{18}O$ produced during the reduction of $KN^{18}O_3$. The second assay measures excreted nitrite resulting from nitrate reduction under anaerobic conditions. Nitrite production in this anaerobic, intact-tissue assay was dependent upon the presence of phosphate (pH 7.5) and was increased by ethanol and bisulfite.

After ten hours of nitrate induction, nitrate reductase activities measured by the $KN^{18}O_3$ assay are one-sixth, and those measured by the anaerobic intact-tissue assay are one-third, of those observed in cell-free extracts of aleurone layers. Addition of ethanol to the anaerobic intact-tissue medium increased the rate of nitrate reduction to a level greater than that found in the cell-free assay.

Oxygen inhibited nitrite release in the anaerobic intact-tissue assay. However, under aerobic conditions and in the presence of 2-heptyl-4-hydroxyquinoline-*N*-oxide or antimycin A, nitrate reduction increased to rates comparable to those observed under anaerobiosis. Neither of these electron transport inhibitors affected anaerobic nitrate reduction, though they were effective in inhibiting oxygen uptake in separate experiments.

It is important to know if enzyme activities observed in cell-free extracts are representative of those which occur in the intact cell or tissue. For nitrate reductase, we have been able to measure its intracellular activity by two different methods, and to compare them with enzyme activity determined in cell-free extracts.

The first method is based on the use of $KN^{18}O_3$ as substrate for nitrate reductase, the rationale of which is as follows. The reduction of nitrate to ammonia is believed to occur via a pathway consisting of four reactions:[1]

$$KN^{18}O_3 \xrightarrow{(NR)} N^{18}O_2{}^- \rightarrow [HN^{18}O] \rightarrow [NH_2{}^{18}OH] \rightarrow NH_4{}^+$$
$$+ \qquad + \qquad + \qquad +$$
$$H_2{}^{18}O \qquad H_2{}^{18}O \qquad H_2{}^{18}O$$

In the first and probably the rate limiting-step in the pathway, nitrate is reduced to nitrite by nitrate reductase. This reaction involves the release of one ^{18}O

atom as $H_2{}^{18}O$. The products of nitrite reduction are not known exactly, but the second and third oxygens of nitrate are released during the final two or three steps of the pathway, or perhaps by nonenzymatic exchange of the $N^{18}O_2^-$, $[HN^{18}O]$ and $[NH_2{}^{18}OH]$ oxygens with oxygens of water. The oxygen atoms of $KN^{18}O_3$ have a very low exchange rate with water (half life measured in years) at all pH values; however, those of nitrite exchange rapidly at low pH values. Thus, one-third of the ^{18}O released as water during the reduction of nitrate to ammonia is indicative of the *in vivo* activity of nitrate reductase. This is true whether the oxygens are enzymatically released from the nitrogen atom, as indicated in the above equation, or in the nonenzymatic exchange with cellular water at the nitrite, or latter, stages of reduction. Thus, the method measures the amount of enzyme involved in nitrate reduction—not necessarily total enzyme levels—in the presence of nitrate only, without addition of any other exogenous materials and under natural (aerobic) conditions.

The second, more rapid intact-tissue assay, is based on the observation[2] that under anaerobic conditions nitrite resulting from nitrate reduction is excreted from the tissue. The method differs from the $KN^{18}O_3$ assay in that assay conditions (anaerobiosis and high phosphate concentrations) are not normally what one might expect *in situ*. Nevertheless, it appears that this second assay technique will be especially useful as a rapid method for studying total levels of nitrate reductase in different biological materials and studying the factors which control the cellular activity of this enzyme.

Barley aleurone layers develop nitrate reductase activity upon exposure to nitrate.[3] We have used isolated aleurone layers to compare nitrate reductase activity of cell-free extracts to enzyme activity observed in the two intact-tissue assays.

Materials and Methods. The procedure used to prepare aleurone layers was similar to that described by Chrispeels and Varner.[4] Barley seeds (*Hordeum vulgare* L. cv. Himalaya) were cut in half, the embryoless halves were imbibed on moist sand, and after 4 to 5 days, the aleurone layers were removed.

Nitrate reductase was induced at 23°C under aseptic conditions in either of two ways:

(1) For the $KN^{18}O_3$ assay, a 5-μl drop of $KN^{18}O_3$ (0.05 *M*) was added to each of 20 aleurone layers. Chloramphenicol (10 μg/ml) was added to the sterilized $KN^{18}O_3$ stock solution to insure against microbial growth during the subsequent induction period. The layers were placed in 5-cm, sterilized, foil-covered Petri dishes. A moistened filter paper (4.25-cm diameter) placed against the lid of the Petri dish prevented dehydration of the tissue. After a 10-hr induction period, water was sublimed (at 80 to 100 μ pressure) from the tissue and collected in a trap cooled with liquid nitrogen. The sublimed water was weighed and placed in 12-ml conical Pyrex tubes containing 46 mg of $NaHCO_3$. The tubes were stoppered with serum vial caps, frozen, and evacuated to approximately 100 μ. The solution was made acidic by injection of 0.2 ml lactic acid, thereby releasing CO_2. After about 24-hr equilibration at 40°C, the tubes were placed in dry ice and the atom per cent excess of $C^{18}O_2$ determined with a MAT GD 150 mass spectrometer. From the weight of extracted water and the isotopic enrichment of CO_2, the number of moles of nitrate reduced were computed.

(2) For the anaerobic intact-tissue assay, nitrate reductase was induced by placing 40–50 aleurone layers and two drops of chloramphenicol (0.5 mg/ml) in a 50-ml Erlenmeyer flask containing 5 ml of sterilized 0.05 *M* KNO_3. The flasks were stoppered with cotton plugs and placed in a metabolic shaker set at 200 rpm. After 2 to 3 hr of induc-

tion the tissue was rinsed with approximately 20 ml of 0.05 M KNO_3, and the effect of various conditions on enzyme activity during the assay was determined. The intact-tissue assay media contained in 2 ml: 10 aleurone layers, 0.1 M phosphate buffer (pH 7.5), 0.02 M KNO_3, and treatment solution as indicated in the text. To start the assay, the reaction mixture, contained in a 25-ml Erlenmeyer flask, was de-aerated by bubbling nitrogen gas through the medium for 1 min, and then stoppered. Nitrite in the media was determined at zero time and after 20 or 30 min of incubation, by adding aliquots to 0.3 ml each of 1% sulfanilamide in 3 N HCl and 0.02% N-1-naphthylethylenediamine dihydrochloride. Optical density at 540 nm was measured after centrifuging at 2000 $\times$ g for 10 min.

Cell-free activity of nitrate reductase was determined as described previously.[3]

Oxygen uptake was measured polarographically with a Clark oxygen electrode. The 2.7-ml reaction media contained 0.02 M KNO_3, 0.1 M phosphate buffer, 10 aleurone layers, and the test substance dissolved in ethanol. Pure ethanol was added to controls.

Results. Comparison of nitrate reduction rates in the intact-tissue and cell-free assays: Enzyme activities as determined by the standard cell-free assay, the anaerobic intact-tissue assay, and the $KN^{18}O_3$ *in vivo* assay are shown in Table 1. Nitrate reductase induced under exactly the same conditions shows 6 and 2.5 times more activity in the cell-free and anerobic intact-tissue methods, respectively, than in the $KN^{18}O_3$ *in vivo* assay.

TABLE 1. *Nitrate reductase activity as determined by the cell-free and intact-tissue assay methods.*

Assay method	Nitrate reductase activity (nmoles NO_3^- reduced/layer·hr)
Cell-free	8.4
Anaerobic intact-tissue	3.2
$KN^{18}O_3$ intact-tissue	1.4

For each assay data were obtained from tissue induced 10 hr under identical conditions by method 1, as described in *Materials and Methods*. Numbers represent averages of three experiments.

TABLE 2. *Dependence of nitrate reductase activity upon components of cell-free assay media.*

	Nitrate reductase activity (nmole NO_2^- formed/layer·hr)	
	Anaerobic intact-tissue assay*	Cell-free assay
Complete	3.6	9.2
Complete-uninduced	0.0	0.0
$-$FMN	3.7	0.0
$-Na_2S_2O_4$	3.2	0.0
$-$phosphate buffer	0.9	2.3
$-KNO_3$	0.3	0.0

* Actually represents anaerobic, intact-tissue assay supplemented with components of cell-free assay. Data from tissue induced 22 hr by method 1 as described in *Materials and Methods*. Numbers represent averages of three experiments.

In an attempt to determine what factor was limiting the rate of nitrate reduction in the intact-tissue, nitrate-induced aleurone layers were assayed in the same medium as used in the cell-free assay. For this, the layers were placed in 2 ml of media containing flavin mononucleotide (FMN), $Na_2S_2O_4$, KNO_3, and phosphate buffer. The reaction was run in the presence of nitrogen to prevent oxidation of the $Na_2S_2O_4$ by air. Under these conditions nitrite was released from the tissue, and the amount of nitrate reductase activity estimated by removing aliquots of the reaction medium after a suitable time period and analyzing for nitrite. Enzyme activity in the complete system, and its dependence on each of the reaction components is shown in Table 2. Nitrate reduction in the intact layers in the presence of $Na_2S_2O_4$, FMN, and inorganic phosphate was greater

than in the $KN^{18}O_3$ *in vivo* assay, but still only approximately one-third the rates observed in the cell-free assay.

Effect of arsenate, ethanol, antimycin A, and HOQNO† on nitrate reduction in the anaerobic intact-tissue assay: From the results presented in Table 2 it seemed that phosphate in some way limits nitrate reduction in the tissue under anaerobic conditions. Though it is less effective, arsenate can substitute for phosphate in enhancing nitrate reduction (Table 3). In later experiments, Tris buffer was also found to be effective; therefore, it is not possible to decide whether the enzyme's response to these compounds is a nonspecific ion or pH effect.

TABLE 3. *Dependency of nitrate reductase activity upon phosphate or arsenate in the anaerobic intact-tissue assay.*

Treatment	Nitrate reductase activity (nmole NO_2^- released/ layer·hr)
$KNO_3 - HPO_4^{--}$	0.3
$KNO_3 + HPO_4^{--}$ (0.01 *M*)	2.7
$KNO_3 + AsO_4^{--}$ (0.01 *M*)	1.9

Tissue induced for 3 hr by method 1 as described in *Materials and Methods*. Numbers represent an average of two experiments. AsO_4^{--} was adjusted to pH 7.5 with NaOH.

TABLE 4. *Effect of ethanol on nitrate reductase in the anaerobic intact-tissue assay.*

Assay condition	Nitrate reductase activity (nmoles NO_2^- released/layer·hr)
N_2 − EtOH	3.6
N_2 + EtOH	16.6
N_2 + EtOH − Tissue	0.0
Air + EtOH	2.0
Air − EtOH	0.1
N_2 + EtOH − P_i	1.0
N_2 + EtOH uninduced	2.5
N_2 − EtOH uninduced	0.3

Tissue induced 3.5 hr by method 2 as described in *Materials and Methods*. Numbers represent average of two experiments.

Ethanol also enhanced nitrate reduction in the anaerobic intact-tissue assay (Fig. 1). Ethanol increases nitrite release under both anaerobic and aerobic conditions. This increased sensitivity for measuring rates of nitrate reduction allowed the detection of low levels of nitrate reductase in noninduced tissues (Table 4). The ethanol-enhanced nitrate reduction was strongly dependent on phosphate buffer (Table 4).

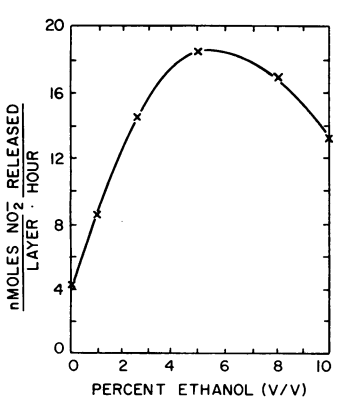
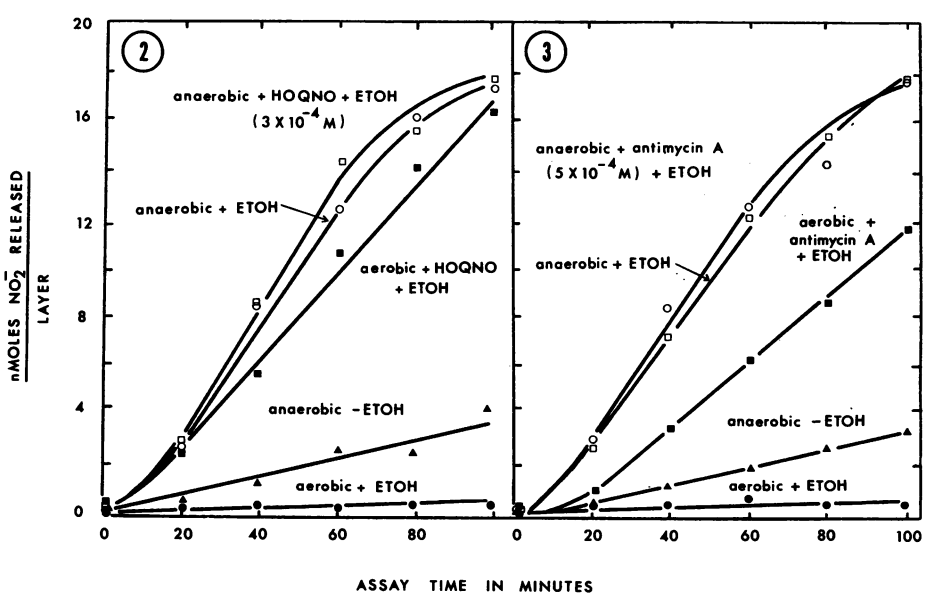


FIG. 1. Concentration curve of ethanol-enhanced nitrite release in the anaerobic intact-tissue assay. Tissue was induced 3 hr by method 2 as described in *Materials and Methods*.

Because bisulfite inhibits the reduction of acetaldehyde during glycolysis, its effect on the anaerobic reduction of nitrate was determined. Results qualitatively similar to those obtained with ethanol were obtained with 10^{-3} *M* bisulfite ($NaHSO_3$). The chloramphenicol normally present as antiseptic in the assay media possesses a potentially sulfite-reactive carboxyl group; therefore, penicillin was added in its place. Also, the pH of the reaction media was reduced from 7.5 to 5.5 to ensure the presence of the reactive HSO_3^- ion species. Neither ethanol nor bisulfite exerted their effect by inhibiting respiration (see next paragraph) because neither chemical inhibited oxygen uptake (Table 5).

The reduction of nitrate under anaerobic conditions and the excretion of the reduced product, nitrite, resembles "nitrate respiration" as described for bacteria[5] and reported to occur in *Vigna sesquipedalis* cotyledons.[2] For this reason the effect of some respiratory inhibitors on nitrate reduction was determined. Both antimycin A and HOQNO, at concentrations which strongly inhibit oxygen uptake (Table 5), had negligible effects on the anaerobic reduction of nitrate (Figs. 2 and 3). However, under aerobic conditions, both these chemicals caused nitrite to be excreted from the tissue (Figs. 2 and 3). The HOQNO and antimycin A effect was not observed with noninduced tissue, and was phosphate dependent. The rate of the aerobic HOQNO-induced nitrite release is nearly identical to that observed under anaerobic conditions (Fig. 2). In the presence of antimycin A, the rate of nitrite release begins to approach rates observed under anaerobic conditions only after a lag of approximately 10 to 15 minutes (Fig. 3). This is consistent with the effect of antimycin A on oxygen uptake: a 15-minute preincubation period is required before oxygen uptake is inhibited 70 per cent (Table 5).

Induction kinetics of nitrate reductase: Figure 4 compares the kinetics of nitrate reductase induction as determined by the intact-tissue and the cell-free assay methods. Included in this figure, for purposes of comparison, is a plot of enzyme activity determined by the anaerobic intact-tissue method in the presence of ethanol. Nitrate reductase activity determined by the $KN^{18}O_3$ assay yields the lowest values of nitrate reduction and is thought to be a correct measure of the rate of nitrate reduction in unmolested tissue. In the anaerobic intact-tissue assay, greater rates of nitrate reduction are observed, but enzyme activity still falls short of that observed in cell-free extracts. However, addition of



FIGS. 2 and 3. Antimycin and HOQNO-enhanced nitrite release under aerobic conditions. Tissue was induced 2-3 hr by method 2 as described in *Materials and Methods.*

ethanol to the anaerobic assay medium causes the rate of nitrate release to exceed nitrite production in cell-free extracts, and is believed to most closely represent total enzyme levels.

Decay kinetics of nitrate reductase: Induction and decay of nitrate reductase activity has been determined using the anaerobic intact-tissue assay to measure enzyme activity (Fig. 5). The assay was performed in the presence of 5 per cent

TABLE 5. *Effect of HOQNO and antimycin on oxygen uptake.*

Treatment	Oxygen uptake (% of control)
Control	100
5% ethanol	135
$NaHSO_3$ (10^{-3} M)*	110
Antimycin A (5×10^{-4} M)	65
Antimycin A (5×10^{-4} M) 15-min preincubation	30
HOQNO (3×10^{-4} M)	40
HOQNO (3×10^{-4} M) 15-min preincubation	10

* At pH 5.5.

The rate of oxygen uptake of ten layers was determined over a period of approximately 5 min, 2–4 min after addition of the test chemical to the reaction media. Chemicals were added in ethanol solutions (final ethanol = 5%). Tissue was induced 2–3 hr by method 1 as described in *Materials and Methods*. Numbers represent an average of two experiments. Phosphate buffer at pH 7.5.

TABLE 6. *Effect of $KN^{18}O_3$ and $KN^{16}O_3$ on nitrate reductase activity.*

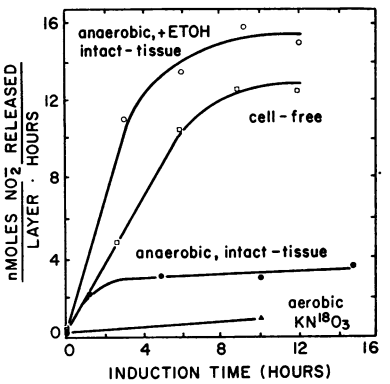
Treatment induced and assayed in:	Activity*
$KN^{16}O_3$	2.4
$KN^{18}O_3$	2.6

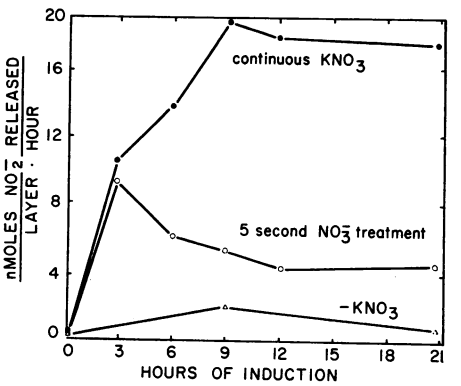
* nmoles NO_2^- released/layer·hr.

Tissue was induced in $KN^{18}O_3$ or $KN^{16}O_3$ and was assayed in $KN^{18}O_3$ or $KN^{16}O_3$, respectively. Enzyme activity was measured with the anaerobic intact-tissue assay. Nitrate reductase was induced by method 1 as described in *Materials and Methods*. Numbers represent an average of two experiments.

ethanol. Only a brief exposure to 0.05 M nitrate is required to induce enzyme activity. The rate of nitrite release begins to decrease 3 hours after nitrate treatment, but there is still measurable nitrate reductase activity after 22 hours. The enzyme activity induced as described in Figure 5 was inhibited 90 per cent by cycloheximide (10 μg/ml) and 20 per cent by 6-methylpurine (2×10^{-4} M) after three-hour induction (both chemicals were present throughout the three-hour incubation period). These kinetic and inhibitor data provide evidence that the ethanol effect is physiological and not a nonenzymatic reduction of nitrate. This experiment also demonstrates the usefullness of the anaerobic intact-tissue assay as a convenient and rapid measure of nitrate reductase activity in plant tissue.

Discussion. A comparison of nitrate reduction rates in the cell-free and in the $KN^{18}O_3$ *in vivo* assay methods indicates that only a small fraction of the total enzyme activity induced in response to nitrate is functioning in the tissue. The low *in vivo* activity is not due to an isotope effect since nitrate reductase induced and assayed in either $KN^{18}O_3$ or $KN^{16}O_3$ (Table 6) showed similar rates of nitrate reduction in the anaerobic intact-tissue assay. The dependence of this latter assay upon phosphate, and the effect of ethanol, suggests that these compounds may, under certain conditions, be involved in the *in vivo* controls of nitrate reduction. For example, phosphate might act indirectly by overcoming the limitation of





(*Left*) FIG. 4. Nitrate reductase induction kinetics as determined by three different assay methods. Tissue was induced by method 1 as described in *Materials and Methods* for the indicated times. $KN^{16}O_3$ was substituted for $KN^{18}O_3$ in all but the isotope assay.

(*Right*) FIG. 5. Decay kinetics of nitrate reductase. In the 5-sec nitrate treatment, 5 ml of 0.05 M KNO_3 was poured over aleurone layers as they were held in a forceps. This treatment was followed immediately by a wash with 40-50 ml of sterile distilled water. The tissue was placed in an additional 40-50 ml water and rinsed once again. The tissue was finally added to 5 ml of sterile distilled water containing two drops of chloramphenicol (0.5 mg/ml). Control tissue was incubated in 5 ml of 0.05 M KNO_3. Throughout the experiment, tissue samples were removed at various time intervals for the assay of nitrate reductase activity.

some phosphate-dependent step in the generation of NADH (e.g., in the oxidation of 3-phosphoglyceraldehyde), or it might act directly with the enzyme. Phosphate has been reported to double the activity of FADH-dependent nitrate reductase isolated from *Neurospora*.[6]

Ethanol may act by indirectly or directly providing reductant for nitrate reduction. Ethanol and several other oxidizable substances have been reported to stimulate nitrate reduction in crude extracts of potato.[7] Reductants generated from aldehydes by isolated potato aldehyde oxidase are capable of reducing nitrate to nitrite.[8] Klepper and Hageman[9] reported stimulation of nitrate reduction by 3-phosphoglyceraldehyde in leaf disks in an *in vivo* assay similar to the anaerobic intact-tissue assay described in this paper.

The enhancement of nitrate reduction by HOQNO and by antimycin A under aerobic conditions suggests that the low activity of nitrate reductase observed in the $KN^{18}O_3$ assay, may be the result of oxygen (via electron transport) competing with the enzyme for reducing equivalents. The fact that antimycin A and HOQNO inhibit oxygen uptake at a site between cytochromes *b* and *c*,[10] taken together with their failure to affect the anaerobic reduction of nitrate, argues against the participation of cytochrome *c* in anaerobic nitrate reduction in this system.

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† Abbreviation: HOQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide.

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