Characterization of the Reversible Inactivation of
Ankistrodesmus braunii Nitrate Reductase by Hydroxylamine

Received for publication March 10, 1986 and in revised form April 29, 1986

TERESA BALANDIN, VICTOR M. FERNÁNDEZ, AND PEDRO J. APARICIO*
Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Velázquez, 144, 28006 Madrid, Spain

ABSTRACT

The photoreversible nature of the regulation of nitrate reductase is one of the most interesting features of this enzyme. As well as other chemicals, NH₂OH reversibly inactivates the reduced form of nitrate reductase from Ankistrodesmus braunii. From the partial activities of the enzyme, only terminal nitrate reductase is affected by NH₂OH. To demonstrate that the terminal activity was reversibly inactivated by NH₂OH, the necessary reductants of the terminal part of the enzyme had to be cleared of dithionite since this compound reacts chemically with NH₂OH. Photoreduced flavins and electrochemically reduced methyl viologen sustain very effective inactivation of terminal nitrate reductase activity, even if the enzyme was previously deprived of its NADH-dehydrogenase activity. The early inhibition of nitrate reductase by NH₂OH appears to be competitive versus NO₃⁻. Since NO₃⁻, as well as cyanate, carbamyl phosphate and azide (competitive inhibitors of nitrate reductase versus NO₃⁻), protect the enzyme from NH₂OH inactivation, it is suggested that NH₂OH binds to the nitrate active site. The NH₂OH-inactivated enzyme was photoreactivated in the presence of flavins, although slower than when the enzyme was previously inactivated with CN⁻. NH₂OH and NADH concentrations required for full inactivation of nitrate reductase appear to be low enough to potentially consider this inactivation process of physiological significance.

In green algae, nitrate reduction has two main physiological purposes: one, assimilatory, to provide the cells with reduced nitrogen to build up living matter for development and proliferation, and the other, regulatory, to keep the internal redox balance using NO₃⁻ as electron sink (4, 5). Thus, the inorganic nitrogen metabolism proved to be highly regulated to accomplish its different tasks. From the different enzymes of this reductive pathway (11), nitrate reductase (NADH-oxidoreductase, EC 1.6.6.1) which reduces NO₃⁻ to NO₂⁻ using reduced pyridine nucleotides as electron donors, is responsible for the main regulatory features that modulate this pathway. Both in vivo and in vitro, this enzyme stands in two stable but reversible forms, active and inactive (2, 5, 11, 22, 29).

NR² from eukaryotes is a large multimeric protein with FAD,

Cyt b₅₅₃ and molybdopterin as electron transport components (8, 22, 23). In addition to its capacity to reduce NO₃⁻ with NAD(P)H (overall activity), the enzyme exhibits two partial activities. The first partial activity is a FAD-dependent NADH-dehydrogenase or diaphorase that reduces a variety of compounds like flavins, DCIP and Cyt c. The second one, is the terminal NR that accepts electrons from reduced flavins or viologens and in which molybdopterin plays an outstanding role. Cyt b₅₅₃, although held in the diaphorase moiety (9), might provide the necessary electron link between these two partial activities (13).

In vitro the reversible inactivation of green algae and higher plant NR was established after the work of Vega et al. (28). At that time, evidence that some reversible inactivating mechanism was also operative in vivo were reported by Herrera et al. (12). To reversibly inactivate NR in vitro, both the absence of NO₃⁻ and reducing conditions were absolutely required. In addition, it was also proved that the presence of chemicals like CN⁻, O₃⁻ and acetylene accelerated the inactivation process (2, 3, 7, 20). The reactivation was readily achieved by ferriyanide, presumably as a consequence of the oxidation of some overreduced state of the molybdenum domain of the enzyme (7, 23, 28). Later, it was found that light promoted the activation of CN⁻-inactivated NR from spinach, and also the activation of NR extracted from Chlorella cells in its inactive form (for review, see Refs. 1 and 21). Evidences of in situ activation of NR by blue light were recently obtained in green algae, particularly in Chlamydomonas reinhardii (4, 5). Hence, both in vivo and in vitro, the photoactivation of eukaryotic NR is an outstanding feature of this enzyme.

Notwithstanding, the in vivo inactivating mechanism still remains a matter of controversy. Recently, the results of the studies of this metabolic event in several nonvacuolated green algae support the presence of a putative N-C metabolite that, especially in the absence of NO₃⁻, would decisively contribute to the in situ reversible inactivation of the enzyme (18). For other authors (24, 29), CN⁻ is still the chemical species that inactivates NR in vivo as it does in vitro. Nonetheless, there are data suggesting that, O₃⁻ might also play an important role for this reversible inactivation of NR, at least in vitro (3, 7).

In an early report Solomonson and Vennesland (26) found that NH₂OH was able to inhibit NR from Chlorella. Hucklesby et al. (14) have further studied this system, establishing that reducing conditions were essential for NH₂OH inactivation of spinach NR. Moreover, Jawali et al. (16) using higher plant enzymes concluded that NH₂OH would impair the electron transport chain of NR mainly by interfering with Cyt b₅₃₅.

Using highly purified Ankistrodesmus braunii NR, we have recently reported (1) that NH₂OH inactivates NR in its reduced state and that the enzyme has a very high affinity for NH₂OH (Kᵣ, 2.1 μM). It was also found that NH₂OH-inactivated NR is
was water from Mm to determinate NR measuring filter from 864 60 when MV was obtained, subsequently were excess through Sephadex of NR purified 7. 60 with chromatography growth the cells were harvested and stored at °C kept available. obtained from Pharmacia, streptomycin sulfate from liver of A. braunii and carbamyl phosphate was obtained from Merck. Sephadex G-25 was obtained from Pharmacia, streptomycin sulfate from Böh- ringen Mannheim. Other reagents were of the purest grade available.

Plant Material. Ankistrodesmus braunii (Nageli) Brunnth (Monoraphidium braunii, Legnerová), strain 202-7c from Göt- tingen University’s Culture Collection was grown under contin- uous light at 25°C on 8 mM KNO3 as described (8). After 5 d of growth the cells were harvested and stored at °C until use.

Purification of NR. NR was purified essentially according to the procedure described by De la Rosa et al. (8). This procedure included as main steps: streptomycin sulfate fractionation and affinity chromatography on Blue Sepharose. Wet Blue Sepharose was added to the supernatant of the streptomycin treatment (0.25 ml/enzymic unit). The suspension was stirred for 30 min and washed to remove unbound protein with 0.1 M KCl and subsequently with standard buffer (20 mM K-phosphate, pH 7, containing 0.15 mM EDTA, and 20 mM FAD). The Blue Sepharose was then packed in a column and NR eluted with 0.5 mM NADH dissolved in the above buffer solution. The purified NR obtained was used as the source enzyme. Ordinarily the enzyme preparations had a specific activity between 20 and 60 units/mg protein. They were kept at °C.

Inactivation and Reactivation of NR. For the inactivation process of NR, samples of the original enzyme preparations were either diluted 10 times with 0.1 M K-phosphate, pH 7, or filtered through Sephadex G-25 and eluted with 0.1 M K-phosphate, pH 7.

For the reactivation process of NR, the incubation mixtures were filtered on Sephadex G-25 equilibrated as above to remove the excess of inactivating agents.

For chemical reactivation, the filtered inactive enzyme was subsequently incubated with different concentrations of ferricyanide. After 2 and 6 min incubation, aliquots were taken out to determine NADH- or MV*-NR activities. The data shown in Table III correspond to the highest ferricyanide reduction values obtained, that in that particular case were achieved with 40 μM ferricyanide.

For light reactivation of NR, the filtered inactive enzyme was irradiated in an open-air glass tube kept at 4°C by a stream of water from a LAUDA thermostated bath. Light (600 μE m-2 s-1) was obtained with an ordinary slide projector fitted with the blue filter from a Oriel color additive separation set.

Enzymic Assays. NADH-NR activity was determined by follow- ing the oxidation of NADH at 340 nm (20). NADH-dehydro- genase activity was assayed by measuring the reduction of DCIP at 600 nm (26). MV*-NR was estimated by two methods. When MV was reduced by Na2S2O4, NR was monitored by colorimetrically measuring NO3- (20). In the experiments in which NR was incubated in the presence of Na2S2O4 and NH2OH, these NO3- measurements were corrected for the NO3- formed by the chemical reaction between these two compounds. When MV was electrochemically reduced, MV*-NR activity was monitored at 30°C by following anaerobically the decrease of MV*+ at 604 nm. The extinction coefficient for MV* applied was 13.9 mM-1 cm-1 (10). To reduce MV electrochemically, 0.14

mm MV in 50 mM K-phosphate, pH 7.5, was stirred over a cathode of Hg at a redox potential of –0.5 V according to Thorneley (27). Samples of 2.5 ml were transferred anaerobically to a spectrophotometer cuvette fitted with a glass stopcock and a rubber septum that allowed us to introduce samples anaerobically with appropriate syringes. Optical measurements were made in a Cary 210 spectrophotometer. One enzymic activity unit corresponds to 1 μmol substrate transformed/min.

Analytical Methods. Protein was determined according to the method of Bradford (6), using BSA as a standard. NO3- was measured by the diazo-coupling colorimetric assay (20). Spectro- photometric measurements were carried out in a Bausch and Lomb Spectronic 2000 spectrophotometer.

RESULTS

As reported elsewhere (1), NH2OH reversibly inactivates purified NR from A. braunii, only when NADH was simultaneously present and NO3- absent. Therefore, it was concluded that only the reduced form of the enzyme had affinity for NH2OH. As shown in Figure 1, NADH concentrations as low as 5 μM produced a substantial inactivation of the overall activity of the enzyme within a preincubation period of 5 min.

We have also previously reported (1) that the inactivating effect of NH2OH on NR appears to be of noncompetitive type versus NO3-. This was the case when the enzyme was preincubated with NADH and NH2OH before adding NO3-. However, when the enzyme was in the presence of both NH2OH and NO3- before adding NADH to complete the reaction mixture, the values of the initial rates of NADH oxidation adjust to the representation of a competitive type, K 0.12 mm (see insert of Fig. 2). As shown in Figure 2, the obtained traces from the reaction mixtures that contain NH2OH and limiting amounts of NO3- blended appreciably with time. After 2 min, the adding of high concentrations of NO3- (10 mM) did not restore enzyme activity as it should be expected from the behavior of a typical competitive inhibition. Hence, NO3- protects the enzyme from NH2OH inactivation but, it cannot overcome this effect once it has occurred.

The observation that the competitive inhibitors versus NO3- like cyanate, azide and carbamyl phosphate (Table I) protected the overall activity against NH2OH inactivation, also suggests

![FIG. 1. Effect of NADH concentration on the NH2OH inactivation of NADH-NR: 2.76 μg of NR in 0.2 ml of 0.1 mM K-phosphate, pH 7, were preincubated at 4°C with 0.1 mM NH2OH and NADH as indicated. At the corresponding times, aliquots from the different preincubation mixtures were taken out to measure NADH-NR activity; 100% activity corresponds to 0.4 unit per ml preincubation mixture.](image-url)
that the enzyme binds NH$_2$OH to the NO$_3^-$ active site. 

To further characterize this reversible inactivation (1), the effects of NH$_2$OH on the different enzymatic activities of NR were studied. Figure 3 shows that both, the overall and terminal activities, were impaired by NH$_2$OH, while the dehydrogenase was essentially unaffected.

The fact that the NO$_3^-$ active site has to be in some reduced state for NH$_2$OH inactivation to occur explains the results of Figure 4. When the enzyme was largely deprived of its dehydrogenase activity through heat treatment and NADH was the reductant, the terminal activity was much less susceptible to NH$_2$OH inactivation. Under these conditions, the residual overall activity of the heated enzyme was completely inactivated by NH$_2$OH. Since electrons from NADH are transferred by the dehydrogenase to the terminal activity, the fraction of the terminal activity that was not affected by NH$_2$OH may correspond to that of the enzyme that was deprived of dehydrogenase activity (Figs. 3 and 4). However, a hasty interpretation from these results might lead to the conclusion that NH$_2$OH inactivates overall activity but not terminal activity. Such a conclusion could easily be drawn if the enzyme preparations had previously lost a great part of the native overall and dehydrogenase activities, but still retaining much of their terminal activity (16).

From the different ways to reduce the terminal part of the dehydrogenase deprived enzyme, the utilization of dithionite is the most popular one. However, dithionite reacts chemically with NH$_2$OH yielding NO$_3^-$ in substantial amounts (data not shown). When NR was incubated with both dithionite and NADH, the degree of inactivation caused by NH$_2$OH was much lower than in the absence of dithionite (Fig. 5). Furthermore, in the absence of NADH, the incubation of the enzyme with dithionite or with both dithionite and MV promoted even lower degrees of inactivation of the overall activity by NH$_2$OH. In all cases, separate incubation of the enzyme with either NH$_2$OH, dithionite, or NADH did not alter appreciably the activity of the
Fig. 4. Requirement of the NADH-dehydrogenase activity for the NADH-dependent \textit{NH}_2\textit{OH} inactivation of NR. NR from the same filtered preparation used in Figure 3 was heated at 45°C for 70 min. The remaining enzymic activities were: 85.4% MV*-NR, 10% NADH-NR, and 17% NADH-dehydrogenase of the corresponding values of the native enzyme. Other experimental conditions to inactivate NR were as in Figure 3 except that FAD was not included in the preincubation mixture. Shown 100% enzyme activities were 0.92, 0.12, and 0.15 unit/ml for the MV*, NADH-NR and NADH-dehydrogenase respectively.

Fig. 5. Dithionite interference in the \textit{NH}_2\textit{OH} inactivation of NADH-NR activity. NR (9.3 \(\mu\)g) in 1 ml of standard buffer was preincubated at 4°C individually with the following chemicals: 4.6 mM Na$_2$S$_2$O$_4$, 0.4 mM MV, 30 \(\mu\)M NADH, and 1 mM \textit{NH}_2\textit{OH} (O); with both Na$_2$S$_2$O$_4$ and NADH (●); with Na$_2$S$_2$O$_4$ plus MV plus \textit{NH}_2\textit{OH} (○); with NADH plus Na$_2$S$_2$O$_4$ plus \textit{NH}_2\textit{OH} (△), or with both NADH and \textit{NH}_2\textit{OH} (▲). At the corresponding times, aliquots from the preincubation mixtures were taken out to measure NADH-NR activity; 100% activity corresponds to 0.33 unit/ml preincubation mixture.

Table II. \textit{NH}_2\textit{OH} Inactivation of the Terminal Activity of NR using Electrochemically Reduced MV as Reductant

<table>
<thead>
<tr>
<th>Additions to MV* Solution</th>
<th>MV*-NR Activity</th>
<th>milliunits</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. NO$_3^-$, enzyme</td>
<td>125.8</td>
<td></td>
</tr>
<tr>
<td>2. Enzyme, NO$_3^-$</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>3. SOD + catalase, enzyme, NO$_3^-$</td>
<td>91.6</td>
<td></td>
</tr>
<tr>
<td>4. SOD + catalase, \textit{NH}_2\textit{OH}, enzyme, NO$_3^-$</td>
<td>1.8</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 6. \textit{NH}_2\textit{OH} inactivation of the terminal activity of an enzyme previously deprived of dehydrogenase activity. Requirement of the reductive reagents of its terminal part. NR from the heated preparation used in Figure 4, was irradiated at 4°C with blue light under anaerobic conditions with 20 mM EDTA, 0.1 mM FMN in the absence (open symbols) or in the presence (closed symbols) of 0.1 mM \textit{NH}_2\textit{OH}. At the corresponding times, aliquots from the preincubation mixtures were taken out to measure MV*-nitrate reductase (○), NADH-NR (△), and NADH-dehydrogenase (●). Shown 100% enzyme activities were 0.76, 0.12 and 0.13 unit/ml for the MV*, NADH-NR, and NADH-dehydrogenase.

There are several ways to overcome the presence of dithionite in the reduction of the terminal part of the enzyme by using other agents to reduce MV and flavins. When NR was added under anaerobic conditions to a cuvette containing both electrochemically reduced MV and NO$_3^-$, it was observed a linear decrease in MV* absorbance, which correlates with the appearance of NO$_2^-$ (data not shown). Table II shows, however, that under anaerobic conditions and in the absence of NO$_3^-$, the incubation of NR with electrochemically reduced MV lead to the inactivation of the terminal activity. The degree of inactivation was greatly reduced when electrochemically reduced MV was previously incubated with SOD and catalase. These results suggest that the traces of NO$_2^-$ that might be present in the reaction mixture, may also impair NR, as it has already been described (3, 7). Nevertheless, a complete and rapid (5 min) inactivation of the enzyme was achieved when anaerobic \textit{NH}_2\textit{OH} was added to this later system. Hence, in this case the inactivation of NR cannot be ascribed to NO$_2^-$, but rather to \textit{NH}_2\textit{OH} itself.

It is well known that EDTA reduces photoexcited flavins and that this system is able to sustain high rates of enzymic nitrate reduction (30). When NR previously deprived of its dehydrogenase activity by heat treatment was incubated with this electron donor system under argon, both the terminal and the residual overall activities were inactivated by \textit{NH}_2\textit{OH} with similar kinetics (Fig. 6). Thus, the inactivation by \textit{NH}_2\textit{OH} should be ascribed to some interaction with the reduced terminal part of the enzyme.

Table III shows, that \textit{NH}_2\textit{OH}-inactivated NR was substantially activated, although not fully, by ferricyanide incubation, as it
REVERSIBLE INACTIVATION OF NITRATE REDUCTASE BY NH2OH

Table III. Gel Filtration and Ferricyanide Effect on NR Inactivated by NH2OH in the Presence of NADH

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Enzymic Activities</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native enzyme</td>
<td>MV*-NR</td>
<td>100</td>
</tr>
<tr>
<td>Inactivated enzyme</td>
<td></td>
<td>19</td>
</tr>
<tr>
<td>Inactivated enzyme, then filtered</td>
<td></td>
<td>13.1</td>
</tr>
<tr>
<td>Inactivated enzyme, then filtered, then incubated with ferricyanide</td>
<td></td>
<td>40</td>
</tr>
</tbody>
</table>

occurred when CN⁻-inactivated NR from other sources were incubated with this oxidant (2, 7, 28). Figure 7 shows that the activating effect of blue light on CN⁻- and NH2OH-inactivated NR, proved also to be very similar, although the photoactivation rate of NH2OH-inactivated NR was slower than that of the CN⁻-inactivated enzyme. Under aerobic conditions and in the presence of 20 μM FAD, blue light irradiation of both CN⁻ and NH2OH-inactivated enzymes caused a substantial recovery of the terminal activity and also an appreciable restoration of the overall activity. During exposure, dehydrogenase activity was, however, inactivated gradually. This inactivation was irreversible and most likely caused by the singlet oxygen generated by photoexcited flavins, as has been extensively discussed elsewhere (21, 22).

DISCUSSION

The CN⁻-inactivation process of NR due to its reversibility has been extensively characterized (29). The reported observation that CN⁻ was bound to the NR extracted from Chlorella in its inactive form was a strong argument in favor of CN⁻ being the chemical species involved in the physiological modulation of NR in green algae (19). It has been proposed pathways for the biosynthesis of CN⁻ in plants, in one of these biosynthetic routes, NH2OH was postulated to be an intermediate (15, 25). The presence of NH2OH in plants is still an open question, but there are data indicating the existence of enzymes that can use NH2OH as substrate (14). Although, it has long been established that NH2OH inhibited NR from Chlorella (26), a similar inhibition in higher plants has been postulated only recently (14, 16, 17). Hucklesey et al. (14) suggested that the concentrations of NH2OH and NADH required to impair significantly the reduction of NO3⁻ in spinach were too high to be of physiological significance. However, our data indicate that 80% NH2OH-inactivation of NR from A. braunii was obtained in a 7 min incubation period in the presence of 5 μM NADH. For the A. braunii NR the estimated Kᵣ for NH2OH was 2.1 μM (1).

It was reported that NH2OH impaired only the overall activity of NR from Amaranthus without affecting any of the partial activities of the enzyme (16), it was then suggested that NH2OH could interact with Cyt b557. Later, the same authors obtained, besides the NH2OH-inactivation of the overall activity, a slight inactivation of the terminal activity of NR from spinach. They concluded that NH2OH would interact with Cyt b557 and also with the terminal part of the enzyme without interfering with the molybdenum domain (17). However, using a highly purified NR from A. braunii we were able to completely inactivate the terminal activity of the enzyme by NH2OH. Actually, the results presented in this paper indicate that, to attain this inactivation the enzyme has to be reduced with an appropriate reducing system like photoreduced flavins or electrochemical reduced MV.

Since NO3⁻ active site is located on the terminal part of the enzyme, the competitive nature of NH2OH inhibition described in Figure 2 is consistent with the suggestion that NH2OH only interacts with the terminal part of the enzyme. The fact that NH2OH inactivation depends on initial concentrations of NO3⁻ and progresses during the enzymic assays (Fig. 2), seems to indicate that NH2OH gradually inactivates the enzyme by occupying, in competition with NO3⁻, the nitrate active site. If this active site happens to be in its reduced state, NH2OH remains tightly bound to the enzyme leading to its definitive inactivation. Also supporting this interpretation is the fact that the competitive inhibitors versus NO3⁻ of NR like cyanate, azide and carbamyl phosphate protect the enzyme from NH2OH inactivation. These protective effects are similar to those obtained for other inactivating compounds, e.g. CN⁻ and acetylene (20, 28). The most plausible interpretation is that the inactivating agents, NH2OH,
CN\textsuperscript{-}, and acetylene, bind strongly to the nitrate active site in its reduced state. CN\textsuperscript{-} apparently forms a one to one stable link with Mo(IV) as it has been detected by electron paramagnetic resonance (23).

Conversely, the activation of the enzyme implies an oxidation of the inactivating agent-enzyme complex (21, 22). The titration of the ferricyanide activation of the CN\textsuperscript{-}-inactivated nitrate reductase showed a highly positive redox midpoint potential, +230 mV for \textit{A. braunii} and +371 mV for \textit{Neurospora} (22) enzymes. NH\textsubscript{3}OH-inactivated NR was, however, only partially activated by ferricyanide, a 20\% activation being reported for higher plants enzymes (14, 17). However, we found in this work a 40\% ferricyanide activation for the \textit{A. braunii} enzyme which suggests that the midpoint potential for this reaction might be very close to that of ferricyanide. The fact that ferricyanide was unable to reactivate acetylene-inactivated NR from spinach might indicate that the midpoint potential for the oxidation of the acetylene-inactivated enzyme was even higher (20). Nevertheless, in all cases tested, inactivated NR was photoactivated by irradiation with blue light presumably by excited flavins.

From this and other previous work (1), it could be concluded that the most significant characteristics of this NH\textsubscript{3}OH inactivation of nitrate reductase are: (a) the interference of NH\textsubscript{3}OH with the enzyme at the nitrate active site in its reduced state; (b) the stability of the inactive enzyme, since the removal of the excess of the inactivating agents by gel filtration did not restore the enzyme activity; and (c) the reversibility of the inactivation which can be achieved by oxidation either chemically or photochemically.

Whether NH\textsubscript{3}OH could play a physiological role in the regulation of NR activity from eukaryotes deserves further investigation. Experiments are in progress to elucidate this point in \textit{A. braunii} cells.

Acknowledgment—The authors thank Ms. C. F. Cabrera for her secretarial assistance.

LITERATURE CITED

1. APARICIO PJ, T Balandin, SG MAUR\textsuperscript{\textcircled{I}}R\textsuperscript{\textcircled{O}}S, JM MALDONADO 1985 Photoregulation of nitrate utilization in green algae and higher plants. Photochem Photobiol 42: 765–770
7. DE LA ROSA MA, C GOMEZ-MORENO, JM VEGA 1981 Interconversion of nitrate reductase from \textit{Antistrodesmus braunii} related to redox changes. Biochim Biophys Acta 662: 77–85
9. FERNANDEZ E, J CARDENAS 1983 Isolation and properties of the NADPH-cytochrome c reductase subunit of \textit{Chlamydomonas reinhardtii} NADPH-nitrate reductase. Biochim Biophys Acta 745: 12–19
12. HERRERA J, A PANIQUE, JM MALDONADO, JL BAREA, M L\textit{O}\textit{S}ADA 1972 Regulation by ammonia of nitrate reductase synthesis and activity in \textit{Chla-
mydomonas reinhardtii}. Biochim Biophys Res Commun 48: 996–1003
14. HUCKLEBRY DP, MJ DOWLING, EJ HEWITT 1981 Metabolism of hydroxyl-
16. JAWALI N, JK SAINIS, PV SANE 1978 Hydroxylamine inhibition of the nitrate reductase complex from \textit{Amaranthus}. Physiologiae Plantarum 17: 1527–1530
17. JAWALI N, PV SANE 1984 Inhibition of the nitrate reductase complex from spinach by oxylamines. Phytorehem 23: 225–228
20. MALDONADO JM, MA VARGAS, SG MAUR\textsuperscript{\textcircled{I}}R\textsuperscript{\textcircled{O}}S, PJ APARICIO 1981 Inactivation by acetylene of spinach nitrate reductase. Biochim Biophys Acta 661: 112–119