Effect of Nitrate on the Synthesis and Decay of Nitrate Reductase of *Neurospora*

By GEORGE J. SORGER, MARIA TERESA DEBANNE and JACQUELINE DAVIES
Departments of Biology and Immunology, McMaster University, Hamilton, Ont., Canada

(Received 6 December 1973)

1. A method was developed to examine the turnover of nitrate reductase by the use of tungstate. 2. Evidence is presented which suggests that the disappearance of nitrate reductase activity from *Neurospora* mycelia exposed to non-inducing conditions is due to the disappearance of the enzyme protein(s) from the mycelia, and not merely due to the disappearance of its (their) catalytic power. 3. The presence of NO_3^- in the culture medium slows down the rate of degradation of nitrate reductase in *Neurospora* *in vivo*.

Nitrate has been shown to be required for the induced formation of nitrate reductase in all fungi studied (Nason & Evans, 1955; Kinsky, 1961; Sorger, 1965; Cove, 1966; Pichinoty & Méténier, 1967; Sims *et al.*, 1968; Lewis & Fincham, 1970), with one exception claimed (Morton, 1956).

When cycloheximide is present in the culture medium, *Neurospora* mycelia accumulate a 'factor X' which behaves like a template for, or a stimulant of, the synthesis of nitrate reductase. 'Factor X' accumulates more quickly, initially, if NO_3^- is present in the culture medium but after 2 h of the cycloheximide treatment the net accumulation of the factor is usually the same whether NO_3^- was present in or absent from the incubation medium. Once 'factor X' has accumulated, the expression of its stimulation or template function in mycelia transferred to media devoid of cycloheximide is profoundly enhanced by the presence of NO_3^- in the medium (Sorger & Davies, 1973). This evidence has been taken to suggest that NO_3^- is not required for the transcription of some critical gene(s) that control(s) the structure of nitrate reductase, but rather that it either stimulates the translation of some critical nitrate reductase messenger RNA species, and/or the correct folding or maturation of the enzyme product, and/or it protects the enzyme from destruction or inactivation.

There is some suggestion that NO_3^- slows down the destruction or inactivation of nitrate reductase in *Neurospora* *in vivo* (Subramanian & Sorger, 1972a; Sorger & Davies, 1973).

The present investigation attempts to answer the following question: is the requirement of NO_3^- for the induction of nitrate reductase in *Neurospora* due entirely to its protection of the enzyme from destruction?

To answer this question, it is essential to establish that the nitrate reductase protein(s) disappear(s) from *Neurospora* mycelia under non-inducing conditions, and it is necessary to measure the rate of disappearance of the enzyme *in vivo* when NO_3^- ions are present in or absent from the culture medium.

Cycloheximide slows down the rate of disappearance of nitrate reductase activity from *Neurospora* mycelia incubated in non-inducing conditions (Subramanian & Sorger, 1972a), from *Ustilago* (Lewis & Fincham, 1970) and from barley (Travis *et al.*, 1969); consequently it is not a generally useful tool for the examination of the effect of NO_3^- ions on the rate of destruction or inactivation of this enzyme.

When *Neurospora* mycelia are incubated in a medium containing tungstate, the mould synthesizes an enzyme that resembles nitrate reductase in its behaviour during purification, in its sedimentation, in its thermal stability, in its inducibility, and in its catalysis of the reduction of cytochrome *c* by NADPH (Subramanian & Sorger, 1972c). The tungstate-poisoned enzyme has lost its capacity to catalyse the reduction of NO_3^- with NADPH and with reduced Benzyl Viologen. Tungstate-poisoned nitrate reductase has been described in higher plants (Wray & Filner, 1970; Notton & Hewitt, 1971) and in algae (Vega *et al.*, 1971), and the tungstate has been shown to be incorporated into the enzyme (Notton & Hewitt, 1971; Paneque *et al.*, 1972) instead of molybdate (Notton & Hewitt, 1971; Aparicio *et al.*, 1971).

The rate of decay of nitrate reductase in *Neurospora* *in vivo* has been studied in the present work in the following way: mycelia induced by NO_3^- were transferred to media containing a high concentration of tungstate under various conditions; any newly formed enzyme should not be able to catalyse the reduction of NO_3^- with NADPH, and it should therefore be possible to follow the fate of the enzyme formed before transfer of the mycelia to conditions of tungstate toxicity.

Two strains were compared: the wild type, which requires the presence of NO_3^- ions in the culture medium for the induction of nitrate reductase, and *nit-3*, which does not require the presence of NO_3^- ions in the culture medium for the de-repressed formation of Benzyl Viologen–nitrate reductase (a partial activity of nitrate reductase; Sorger, 1966).

Materials and Methods

Strains

Neurospora strain 3.1a (fungal stock no. 988: Fungal Stock Center, California State University, Humboldt, Arkata, Calif., U.S.A.) was used as the wild-type strain. Strain *nit-3* (fungal stock no. 358) lacks NADPH-nitrate reductase and NO_3^- -inducible NADPH-cytochrome *c* reductase, but retains one of the partial activities of nitrate reductase, i.e. Benzyl Viologen-nitrate reductase. Unlike the wild-type strain, however, this partial activity is not induced by the presence of NO_3^- ions in the incubation medium of the mould, but like the wild-type strain the partial activity is repressed by the presence of NH_4^+ ions in the culture medium (Sorger, 1966). The mutant grows on NO_2^- , NH_4^+ and on hypoxanthine as N sources, but not on NO_3^- . Strain *DJ-2* is a mutant that is temperature-sensitive with respect to NO_3^- utilization; it has recently been isolated (D. A. Jones & G. J. Sorger, unpublished work) in our laboratory from a pantothenic acid-requiring parent (fungal stock no. 1707). Strain *DJ-2* appears to be allelic with *nit-3*; it grows normally on NO_2^- , NH_4^+ and on hypoxanthine as N sources, and grows slowly on NO_3^- as sole source of N at the permissive temperature only; the mutant produces an NADPH-nitrate reductase with all its associated partial activities when it is induced by NO_3^- at the permissive temperature (G. J. Sorger & J. Davies, unpublished work).

Culture conditions

The basic medium without a N source was as described by Sorger & Giles (1965) and contained glucose, macro and micro mineral elements. In addition, the media contained one of the following N sources unless otherwise stated: ammonium tartrate, 4g/l (NH_4^+ medium); NaNO_3 , 5g/l (NO_3^- medium). Agar (1.5%; Difco) was added when solid media were desired. The detailed growth, induction and repression procedures are described in the legends to each figure.

Extractions

Enzyme preparations were made as described by Sorger & Davies (1973), except for those which were expected to have very little nitrate reductase activity. The latter were extracted in one-half of the usual volume of buffer per mycelial pad.

Enzyme and protein assays

NADPH-nitrate reductase and Benzyl Viologen-nitrate reductase were assayed as described by Subramanian & Sorger (1972c). One unit of activity is defined as the production of 1nmol of NO_2^- /min

at $30 \pm 1^\circ\text{C}$. Specific activity is expressed as units/mg of protein. When the specific activity of a preparation was lower than 5, the incubation time of the assay was increased from the customary 15min to obtain a reliable measurement. In each case where this was done, proportionality between the amount of product formed and time of incubation, and between activity and extract concentration, was checked.

The concentration of protein was measured by the biuret method (Dawson *et al.*, 1959) with bovine serum albumin as reference.

Preparation of antibodies

Extracts of induced wild-type mycelia were layered on top of 5ml linear 5–20% (w/v) precooled gradients of sucrose in 0.1M-sodium phosphate buffer, pH7.0, and centrifuged at 117000g for 13h at 5°C in a swinging-bucket rotor (Spinco SW 50.1). Fractions (approx. 0.16ml each) were collected from the gradients with the aid of gravity by piercing the bottom of the centrifuge tube with a hypodermic needle. A sample (0.5ml, containing approx. 3 units of NADPH-nitrate reductase activity) of the pooled fractions was mixed with 0.5ml of Freund's adjuvant and injected subcutaneously into rabbits at weekly intervals for 3 successive weeks. On the fourth week, the final injection was given intravenously without adjuvant, and the rabbits were bled 1 week later. The whole blood was allowed to clot, and the sera were stored at -18°C in 0.5ml batches; 4 units of nitrate reductase activity were 50% inactivated by 0.04ml of the antiserum. Normal serum, taken from the rabbits before the immunization schedule, did not inhibit nitrate reductase activity.

Determination of equivalence point

Increasing quantities of crude extract made up to a total of 0.55ml with 0.1M-potassium phosphate buffer, pH7.0, were added to 0.05ml of antiserum. The mixture was incubated for 1h at 4°C and the activity remaining was measured by adding the entire mixture to 1.75ml of substrate solution (0.2M-sodium pyrophosphate buffer, pH7.0–0.1M- NaNO_3 –1mM-FAD; 50:20:1, by vol.) and starting the reaction with 0.25ml of 2mM-NADPH. The assay mixture was incubated for 15, 30 or 60min depending on the activity of the extract, and the reaction terminated by adding 0.05ml of 25% (w/v) barium acetate followed by 2.5ml of 95% (v/v) ethanol. Nitrite was measured colorimetrically in the usual fashion (Nason & Evans, 1955). One unit of nitrate reductase activity is as defined above.

The equivalence point is defined as the minimum number of units of nitrate reductase activity that must be added to 0.05ml of the antiserum to have an excess of antigen in the reaction mixture described above.

Materials

Cycloheximide, NADPH, FAD and bovine serum albumin were from Sigma Chemical Co., St. Louis, Mo., U.S.A.; Benzyl Viologen was from Schwarz-Mann Co., Orangeburg, N.Y., U.S.A.; all other chemicals were of reagent grade and were from Fisher Scientific Co., Fairlawn, N.J., U.S.A. or from Baker Chemical Co., Phillipsburg, N.J., U.S.A.

Results and Discussion

Is the disappearance of nitrate reductase activity from mycelia incubated under non-inducing conditions caused by the disappearance of the enzyme protein(s)?

Mycelia that are removed from contact with NO₃⁻ quickly lose their nitrate reductase activity; if they are subsequently re-exposed to NO₃⁻ the enzymic activity reappears, and this reappearance is blocked by cycloheximide (Table 1). This situation resembles that described by Oaks *et al.* (1972) in corn, and suggests that the reappearance of nitrate reductase in these cases requires protein synthesis.

Fig. 1 shows that the equivalence point of wild-type nitrate reductase-anti-(wild-type nitrate reductase) antibody (i.e. the intercept of the line with the x axis)

Fig. 1. Effect of time of incubation of mycelia under non-induction conditions on the equivalence point of nitrate reductase - anti-(wild-type nitrate reductase) antibody from cell-free extracts

Table 1. Effect of cycloheximide on the reinduction of nitrate reductase in mycelia that have been exposed to non-inducing conditions

Wild-type mycelial pads, pregrown on NH₄⁺ medium, were induced for 4h on basic medium containing 5mm-NaNO₃, and subsequently incubated (post-induction treatment) for 2h on basic medium without a N source. Some of the pads were harvested, and others were incubated on basic medium containing one of the following: 3mm-NaNO₃, 3mm-NaNO₃ plus 1μg of cycloheximide/ml, 10mm-ammonium tartrate, or no N source. The mycelia were harvested, extracted and the cell-free preparations assayed immediately for NADPH-nitrate reductase activity. The concentration of protein in the cell-free extracts was between 5.1 and 6.3mg/ml. The results of this experiment are representative of two sets of determinations.

Post-induction treatment	Nitrate reductase specific activity (units/mg of protein)
2h with no N source	1.1
2h with no N source followed by 2h on NaNO ₃	10.1
2h with no N source followed by 2h on ammonium tartrate	0.5
2h with no N source followed by 2h no N source	0.4
2h with no N source followed by 2h on NaNO ₃ plus cycloheximide	0.6

Wild-type mycelial pads were pregrown for 44h on NH₄⁺ medium, and subsequently induced for 4h on basic medium containing 20mm-NaNO₃. Some pads were collected, extracted and used immediately (○); others were transferred to basic medium containing 10mm-ammonium tartrate and incubated for 1h (●) or 2h (△) and then harvested. Mycelia were then extracted and the equivalence point was measured as described in the Materials and Methods section. The specific activities of the extracts were 18.5, 9.5, and 4.5 for the preparations of mycelia exposed to NH₄⁺ for 0, 1 and 2h respectively. The total protein extracted/mycelial pad was between 9 and 18mg. Mycelial pads of strain DJ-2 were pregrown, induced and extracted immediately as described above (▼). The specific activity of the extract was 1.2. The results of the experiment with the wild-type strain are representative of those from three other experiments, all of which gave an equivalence point between 2 and 2.5 units. The results with the extract of strain DJ-2 are representative of two sets of determinations.

is the same in extracts of fully induced mycelia and in extracts of mycelia that have been induced and subsequently exposed to non-inducing conditions for different lengths of time. The equivalence point is

sensitive to changes in the conformation of an antigen, as shown by the observation that it is different in preparations of the wild-type strain and in preparations of strain *DJ-2* (Fig. 1). The latter is probably mutated at the structural locus of nitrate reductase (G. J. Sorger, M. T. Debanne & J. Davies, unpublished work). Mutants having an altered nitrate reductase that cross-reacts with antibodies against the wild-type enzyme have been described in *Aspergillus* (Pateman *et al.*, 1964).

If nitrate reductase were partially inactivated, or its conformation were changed in some other way, yet the enzyme retained some antigenic recognition sites, the equivalence point would be changed.

The observation that the equivalence point of wild-type nitrate reductase in extracts containing different specific activities of the enzyme is constant can be interpreted most simply in this case to mean that these extracts contain different quantities of antigenically identical enzyme. Thus it would appear from the above that the disappearance of nitrate reductase

from wild-type mycelia incubated in non-inducing conditions is due to the disappearance of the enzyme protein(s) itself. This seems to be the case in tobacco cells (Zielke & Filner, 1971), but not in algae (Losada *et al.*, 1970; Rigano, 1971), where the reductase is apparently reversibly inactivated under such conditions.

Effect of tungstate on nitrate reductase

The presence of 6 mM-tungstate in the culture medium inhibits the induced appearance of nitrate reductase by approx. 90% in the strains examined (Fig. 2). Higher concentrations of tungstate result in a greater inhibition of the induction of this enzyme activity, but they also inhibit the growth of the organism on NH_4^+ as N source, i.e. non-specifically.

Extracts of tungstate-poisoned mycelia do not seem to inhibit the homologous normal nitrate reductase activity significantly (Fig. 3). It should therefore be possible to obtain reliable estimates of the content

Fig. 2. Effect of tungstate and different N sources on the induction of nitrate reductase

Mycelial pads, pregrown for 44 h on NH_4^+ medium, were transferred to basic medium containing one of the following: 20mM- NaNO_3 (○), 20mM- NaNO_3 plus 6mM- Na_2WO_4 (●), 10mM-ammonium tartrate (▽), 10mM-ammonium tartrate plus 20mM- NaNO_3 (□), no N source (Δ), 20mM- NaNO_3 plus 1 μg of cycloheximide/ml (▲), and incubated with shaking at 27°C for the indicated times. Mycelia were then harvested, extracted and assayed for NADPH-nitrate reductase or Benzyl Viologen-nitrate reductase activity. Each measurement was performed in duplicate and is shown as such on the figure. Where there is only one point, both duplicates coincided. Figs. 2(a) and 2(b) represent observations on the wild-type strain and on strain *nit-3* respectively. In Fig. 2(a) the left-hand ordinate refers to the induction on NO_3^- and the right-hand ordinate refers to induction or de-repression on NO_3^- and on no N source, and the right-hand ordinate refers to induction or de-repression under other conditions. Each experiment is representative of two sets of determinations.

Fig. 3. Effects of extracts of tungstate-poisoned mycelia on the assay of nitrate reductase

Mycelial pads, pregrown for 44 h on NH_4^+ medium, were transferred to basic medium containing either 20 mM- NaNO_3 plus 6 mM- Na_2WO_4 (●), or 10 mM-ammonium tartrate plus 6 mM- Na_2WO_4 (▼) or 10 mM-ammonium tartrate (▽) and shaken at 27°C for 4 h. Cell-free preparations were made of these mycelia and a constant amount of extract from each was added to increasing amounts of extract of fully induced healthy mycelia in nitrate reductase reaction mixtures. Figs. 3(a) and 3(b) represent the observations with the wild-type strain and with strain *nit-3* respectively. The amounts of protein from extracts of mycelia exposed to NO_3^- plus tungstate, NH_4^+ plus tungstate, and NH_4^+ above, which were present in the reaction mixtures of the experiment shown in Fig. 3(a), were 0.33 mg, 0.35 mg and 0.36 mg respectively; in Fig. 3(b) they were 0.39 mg, 0.40 mg and 0.41 mg respectively. Each experiment is shown as having been done in duplicate and represents another that is not shown.

of nitrate reductase activity in tungstate-poisoned mycelia. There is some variability in the measurements shown in Fig. 3. This is usual with the assay of Benzyl Viologen-nitrate reductase, which we cannot make less variable.

The rate of disappearance of nitrate reductase activity in mycelia exposed to NH_4^+ ions or to nitrogen-deficient conditions is not noticeably affected by the presence of tungstate in the culture medium (Figs. 4a and 4b), suggesting that the presence of the inhibitor does not interfere with the decay process itself under these conditions. There appears to be some exchange between molybdate and tungstate on nitrate reductase which does not require protein synthesis (Fig. 5, activation of tungstate-poisoned nitrate reductase by excess of molybdate). This exchange is apparently quantitatively less important an influence on the rate of decay than the experimental error.

The above considerations suggest that tungstate at a concentration of 6 mM in the culture medium is a useful tool for the study of the decay and turnover of nitrate reductase.

Effect of NO_3^- on the synthesis and decay of nitrate reductase

The rate of disappearance of nitrate reductase activity from wild-type mycelia incubated in the

presence of NO_3^- is faster when tungstate is also present in the medium than when tungstate is absent (Figs. 4a and 4b). This indicates that nitrate reductase turns over in wild-type mycelia exposed to NO_3^- . The turnover of nitrate reductase has been reported in tobacco cells cultured in the presence of NO_3^- by Zielke & Filner (1971), where the fate of the enzyme was followed with the aid of ^{15}N .

There is a 6–12-fold difference between the rate of formation of nitrate reductase by wild-type mycelia exposed to culture media containing NO_3^- and by wild-type mycelia cultured in media containing no N source (Fig. 2a, Table 2). The rate of formation of the enzyme by mycelia incubated with NH_4^+ is virtually nil.

The rate of disappearance of nitrate reductase from wild-type mycelia incubated with no N source and tungstate is between 2 and 5 times faster than in wild-type mycelia incubated with NO_3^- and tungstate, and about 1½ times as fast as it is in wild-type mycelia incubated with NH_4^+ ions and tungstate in the culture medium (Fig. 4b).

The situation in *nit-3* is different; NO_3^- is not required for the formation of Benzyl Viologen-nitrate reductase (Fig. 2b) and the rate of decay of the enzyme *in vivo* in the presence of tungstate is relatively insensitive to differences in the N source of the mycelia (Fig. 4c; Table 2).

Fig. 4. Decay of nitrate reductase activity

Fully induced or de-repressed mycelia were transferred to basic medium containing one of the following: 20mM- NaNO_3 ($\circ$); 20mM- NaNO_3 plus 6mM- Na_2WO_4 ($\bullet$); no N source (Δ); no N source plus 6mM- Na_2WO_4 ($\blacktriangle$); ammonium tartrate at 10mM for wild-type mycelia (∇) or at 60mM for strain *nir-3*; 10mM- or 60mM-ammonium tartrate plus 6mM- Na_2WO_4 ($\blacktriangledown$); 10mM- or 60mM-ammonium tartrate plus 20mM- NaNO_3 ($\square$); 10mM- or 60mM-ammonium tartrate plus 20mM- NaNO_3 plus 6mM- Na_2WO_4 ($\blacksquare$). Fig. 4(a) shows the results of an experiment done with the wild-type strain in the absence of tungstate, Figs. 4(b) and 4(c) represent the results of experiments with the wild-type and *nir-3* strains respectively, in the presence of tungstate. Mycelial pads were pregrown on NH_4^+ medium and then induced for 4h on basic medium containing 20mM- NaNO_3 (wild-type strain) or de-repressed for 4h on a medium with no N source (*nir-3*). The induced or de-repressed mycelial pads were then transferred to basic medium containing one of the N sources indicated above for the times shown, harvested, extracted, and the cell-free preparations were assayed for NADPH-nitrate reductase or Benzyl Viologen-nitrate reductase. The total protein extracted/mycelial pad was between 9 and 20mg. Each experiment is representative of at least two others. Duplicate measurements are shown at each point; where these coincide, there is only one symbol.

Neither the rate of formation of nitrate reductase nor the rate of disappearance of the enzyme *in vivo* is constant during induction: wild-type mycelia pre-incubated with NO_3^- and 6mM-tungstate for 1 or 3h and subsequently transferred to a medium containing NO_3^- and 6mM-molybdate synthesize active nitrate reductase about twice as fast as mycelia which were transferred in the above fashion after 6h of pre-incubation with NO_3^- and tungstate (Fig. 5). There seems to be some activation without new protein synthesis of the inactive enzyme formed in the presence of tungstate. This is shown by control mycelia which were transferred from media containing NO_3^- and tungstate to media containing NO_3^- , molybdate and cycloheximide (Fig. 5). This apparent activation of pre-existing, if inactive, enzyme was over after 15min; thus the velocity of synthesis of the newly formed enzyme which is quoted above was that after the first 15min of incubation of the tungstate-

poisoned mycelia in the detoxifying (i.e. NO_3^- - and molybdate-containing medium) conditions (Fig. 5).

The rates of formation of nitrate reductase shown in Table 2 are taken from between 1½ and 4h of induction, which appears to be a time of fairly constant velocity of synthesis of the enzyme (cf. Fig. 5).

The rate of decay of nitrate reductase in mycelia incubated in media containing NO_3^- increases with the time of exposure to NO_3^- (Fig. 6). When wild-type mycelia have been induced on NO_3^- -containing media for 2.5h and subsequently transferred to media containing NO_3^- plus tungstate, the rate of decay of the reductase is about three-quarters of what it is when the mould has been induced on NO_3^- -containing media for 4 or 8h before transfer to media containing NO_3^- plus tungstate (Fig. 6a). The velocity of decay of the enzyme in mycelia induced on NO_3^- -containing media and subsequently transferred to media containing NH_4^+ plus tungstate seems to

be unaffected by the length of time of the previous induction on NO₃⁻-containing media (Fig.

Fig. 5. Effect of the time of induction on the rate of formation of nitrate reductase

Mycelial pads of the wild-type strain, pregrown for about 40h on NH₄⁺ medium, were washed with water and transferred to basic medium containing 20mM-NaNO₃ plus 6mM-Na₂WO₄ and shaken for 1 (▼, ▽), 3 (○, ●) or 8 h (□, ■) at 27°C. At each of the above times, groups of pads were transferred to basic medium containing 20mM-NaNO₃ plus 6mM-Na₂MoO₄ (▼, ●, ■) or 20mM-NaNO₃ plus 6mM-Na₂MoO₄ plus 1 µg of cycloheximide/ml (▽, ○, □), and incubated as above for the indicated times, when the mycelia were harvested, cell-free extracts made, and the preparations assayed for NADPH-nitrate reductase activity. This experiment was repeated with very similar results.

6b). We have no explanation for this puzzling observation. The observations shown in Fig. 6 should be taken into account when interpreting data in Table 2.

The specific activity of nitrate reductase rises for the first 4h during induction and subsequently drops (Fig. 2). This is probably due to the decrease in the rate of synthesis of the enzyme with the time of exposure to inducing conditions (Fig. 5), combined with the increase in the rate of decay of the reductase with this type of exposure (Fig. 6).

The enormous difference in the rates of formation of nitrate reductase by mycelia incubated with NH₄⁺ ions and by mycelia incubated with NO₃⁻ ions is too great to be accounted for by differences in stability of nitrate reductase *in vivo* under these two conditions.

The 6–12-fold difference observed in the rate of synthesis of nitrate reductase by wild-type mycelia cultured in NO₃⁻-containing media and by wild-type mycelia cultured in media containing no N source seems greater than the two- to five-fold difference in the rate of decay of the enzyme in wild-type mycelia incubated under these two conditions. If one applies a correction factor to account for the observations in Fig. 6, then there is a 2.7–6.7-fold difference between the rate of decay of nitrate reductase in wild-type mycelia incubated in the presence of NO₃⁻ and of no N source. In strain *nit-3* both the rates of decay and the rates of synthesis of the enzyme are similar when the mycelia are incubated in media containing NO₃⁻ and/or no N source.

It appears from the above that an important part of the effect of NO₃⁻ on the induction of nitrate reductase in *Neurospora* is its stabilization of the enzyme, but one cannot say with any degree of certainty that the partial protection of the reductase *in vivo* by NO₃⁻ is sufficient to explain the induction

Table 2. Summary of the effect of different N sources on the synthesis and on the decay of nitrate reductase *in vivo*

The rates of formation and of disappearance of nitrate reductase given below are the averages of the slopes of the corresponding curves of all the experiments represented by those depicted in Figs. 2 and 4. Abbreviations: n.d., experiment not done; tr, trace of specific activity (less than 0.03). When the rates from fewer than four experiments are reported, the individual slopes are given; when more than four, the means ±s.e. are shown.

Strain	N source in medium	Rate of formation (Δ specific activity/h)	Maximum specific activity	Rate of disappearance (Δ log of specific activity/h)	
				In the presence of Na ₂ WO ₄	In the absence of Na ₂ WO ₄
Wild-type	NO ₃ ⁻	9.3 ± 0.5	16.2 ± 2.5	0.20 ± 0.06	0.03 ± 0.10
	-N	1.2 ± 0.4	1.3 ± 0.2	0.60 ± 0.10	0.60 ± 0.03
	NH ₄ ⁺	0	tr	0.40 ± 0.03	0.40 ± 0.06
	NH ₄ ⁺ + NO ₃ ⁻	0.5 ± 0.2	1.2 ± 0.4	0.28, 0.35	0.10, 0.15
<i>nit-3</i>	NO ₃ ⁻	15.0, 18.0	50, 37	0.25 ± 0.06	n.d.
	-N	11.0	27	0.29 ± 0.02	n.d.
	NH ₄ ⁺	0	0	0.35 ± 0.02	n.d.
	NH ₄ ⁺ + NO ₃ ⁻	0	0	0.30, 0.30, 0.32	n.d.

Fig. 6. Effect of the time of exposure of wild-type mycelia to inducing conditions, before being transferred to media containing NO_3^- plus tungstate or ammonium tartrate plus tungstate, on the rate of decay of nitrate reductase

Mycelial pads of the wild-type strain were pregrown for about 40 h on an NH_4^+ medium, then washed with water and induced for 2.5 h (▽), 4 h (●) or 8 h (○) in basic medium containing 20 mM- NaNO_3 . The pads were washed with water and transferred for the indicated time to basic medium containing 6 mM- Na_2WO_4 and 20 mM- NaNO_3 (Fig. 6a) or 6 mM- Na_2WO_4 and 20 mM-ammonium tartrate (Fig. 6b). Extracts of the mycelia were subsequently made and assayed for NADPH-nitrate reductase activity. The total extracted protein/mycelial pad was between 9 and 18 mg. Each experiment was repeated at least once with very similar results.

of the enzyme by this species of ion. It also seems that one cannot account for the effect of NH_4^+ on the induction of nitrate reductase by an effect of these ions on the stability of the enzyme *in vivo*.

Mutant DJ-2 was isolated in this laboratory by Mr. David A. Jones. This investigation was supported by the National Research Council of Canada (grant no. A-3649).

References

- Aparicio, P. J., Cárdenas, J., Zumft, W. G., Vega, J. M., Herrera, J., Paneque, A. & Losada, M. (1971) *Phytochemistry* **10**, 1487-1495
- Cove, D. J. (1966) *Biochim. Biophys. Acta* **113**, 51-56
- Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1959) *Data for Biochemical Research*, p. 84, Clarendon Press, Oxford
- Kinsky, S. C. (1961) *J. Bacteriol.* **82**, 898-904
- Lewis, C. M. & Fincham, J. R. S. (1970) *J. Bacteriol.* **103**, 55-61
- Losada, M., Paneque, A., Aparicio, P. J., Vega, J. M., Cárdenas, J. & Herrera, J. (1970) *Biochem. Biophys. Res. Commun.* **38**, 1009-1015
- Morton, A. G. (1956) *J. Exp. Bot.* **7**, 97-112
- Nason, A. & Evans, H. J. (1955) *Methods Enzymol.* **2**, 411-415
- Notton, B. A. & Hewitt, E. J. (1971) *Biochem. Biophys. Res. Commun.* **44**, 702-710
- Oaks, A., Wallace, W. & Stevens, D. (1972) *Plant Physiol.* **50**, 649-654
- Paneque, A., Vega, J. M., Cárdenas, J., Herrera, J., Aparicio, P. J. & Losada, M. (1972) *Plant Cell Physiol.* **13**, 175-178
- Pateman, J. A., Cove, D. J., Rever, B. M. & Roberts, D. B. (1964) *Nature (London)* **201**, 58-60
- Pichinoty, E. & Méténier, G. (1967) *Ann. Inst. Pasteur Paris* **112**, 701-711
- Rigano, C. (1971) *Arch. Mikrobiol.* **76**, 265-276
- Sims, A. P., Folkes, B. F. & Bussey, A. H. (1968) in *Recent Aspects of Nitrogen Metabolism in Plants* (Hewitt, E. J. & Cutting, C. V., eds.), pp. 91-114, Academic Press, New York
- Sorger, G. J. (1965) *Biochim. Biophys. Acta* **99**, 234-245

- Sorger, G. J. (1966) *Biochim. Biophys. Acta* **118**, 484–494
- Sorger, G. J. & Davies, J. (1973) *Biochem. J.* **134**, 673–685
- Sorger, G. J. & Giles, N. H. (1965) *Genetics* **52**, 777–788
- Subramanian, K. N. & Sorger, G. J. (1972a) *J. Bacteriol.* **110**, 538–546
- Subramanian, K. N. & Sorger, G. J. (1972b) *J. Bacteriol.* **110**, 547–553
- Subramanian, K. N. & Sorger, G. J. (1972c) *Biochim. Biophys. Acta* **256**, 533–543
- Travis, R. L., Jordan, W. R. & Huffaker, R. C. (1969) *Plant Physiol.* **44**, 1150–1156
- Vega, J. M., Herrera, J., Aparicio, P. J., Paneque, A. & Losada, M. (1971) *Plant Physiol.* **48**, 294–299
- Wray, J. L. & Filner, P. (1970) *Biochem. J.* **119**, 715–725
- Zielke, H. R. & Filner, P. (1971) *J. Biol. Chem.* **246**, 1772–1779