Biochem. J. (1973) **134**, 673–685
Printed in Great Britain

Regulation of Nitrate Reductase of *Neurospora* at the Level of Transcription and Translation

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(*Received* 29 *December* 1972)

The presence of nitrate is required for the induced synthesis of NADPH–nitrate reductase and its related partial activity Benzyl Viologen–nitrate reductase in a wild-type strain of *Neurospora*. In *nit*-3, a mutant lacking complete NADPH–nitrate reductase activity but retaining the partial activity Benzyl Viologen–nitrate reductase, the presence of nitrate ions is not required for the de-repressed synthesis of the latter enzyme. The accumulation of the capacity to synthesize nitrate reductase, and the related Benzyl Viologen–nitrate reductase, in the absence of protein synthesis does not require nitrate in the normal strain or in strain *nit*-3. Ammonia antagonizes the accumulation of this capacity in both strains. Nitrate is required for the synthesis of nitrate reductase and related activities from presumedly preformed mRNA in the wild-type strain. Nitrate is not required for the comparable function in strain *nit*-3. Ammonia appears to stop the synthesis of nitrate reductase and related activities from presumedly preformed mRNA in the wild-type strain and in strain *nit*-3. The effects of nitrate, or ammonia and of no nitrogen source on the induced synthesis of nitrate reductase cannot be explained on the basis of the effects of the different nitrogen sources on general synthesis of RNA or of protein.

Nitrate has been shown to stimulate or to be required for the appearance of nitrate reductase activity in most higher plants studied (Filner, 1966; Ingle *et al.*, 1966; Hewitt *et al.*, 1967; Schrader *et al.*, 1968; Joy, 1969; Wray & Filner, 1970), with one claimed exception (Bayley *et al.*, 1972*a*,*b*), in most fungi (Evans & Nason, 1953; Silver, 1957; Kinsky, 1961; Sorger, 1965; Cove, 1966; Pichinoty & Méténier, 1967; Sims *et al.*, 1968; Lewis & Fincham, 1970*b*), with one exception claimed (Morton, 1956), and in some eukaryotic algae (Rigano, 1971; Smith & Thompson, 1971*a*,*b*; Thacker & Syrett, 1972) but not apparently in others (Vega *et al.*, 1971; Zumft *et al.*, 1972).

The presence of ammonia in the culture medium has been shown to lower the induced appearance of nitrate reductase activity in some higher plants (Joy, 1969), but not in others (Filner, 1966; Ingle *et al.*, 1966), in all fungi studied (Morton, 1956; Silver, 1957; Kinsky, 1961; Cove, 1966; Sims *et al.*, 1968; Lewis & Fincham, 1970*b*) and in eukaryotic algae (Losada *et al.*, 1970; Smith & Thompson, 1971*a*,*b*; Rigano, 1971; Thacker & Syrett, 1972).

The nitrate-dependent, nitrate-stimulated and/or ammonia-antagonized appearance of nitrate reductase activity has been shown to require protein synthesis or RNA synthesis or both in higher plants (Ingle, 1968; Stewart, 1968; Smith & Thompson, 1971*a*,*b*), in fungi (Sorger, 1965; Lewis & Fincham, 1970*b*; Subramanian & Sorger, 1972*a*,*b*) and in eukaryotic algae (Losada *et al.*, 1970; Rigano, 1971; Smith & Thompson, 1971*a*,*b*).

In one plant system, the nitrate reductase itself has been shown to be made *de novo*, at least in part (Zielke & Filner, 1971), in contrast with a previous report (Ingle, 1968).

Because of the above considerations, nitrate is generally thought to act as co-inducer of nitrate reductase synthesis in the sense of the model of repression proposed by Jacob & Monod (1961). Ammonia or some other end product of nitrate reduction is generally thought to be a co-repressor of nitrate reductase synthesis in eukaryotic organisms.

The intent of this investigation was to examine the effect of nitrate and of ammonia on the transcription and on the translation of nitrate reductase mRNA, by using a modification of a procedure developed previously (Subramanian & Sorger, 1972*b*). A wild-type strain of *Neurospora*, which requires the presence of nitrate ions for the induced synthesis of NADPH–nitrate reductase and of partial nitrate reductase activities, was compared with strain *nit*-3, a mutant that does not require the presence of nitrate ions for the de-repressed synthesis of Benzyl Viologen–nitrate reductase, a partial activity of NADPH–nitrate reductase (Sorger, 1966). Ammonia represses the induced or de-repressed formation of the respective enzyme(s) in both strains (Subramanian & Sorger, 1972*a*).

Materials and Methods

Organisms

Strains. Wild-type *Neurospora crassa*, strains 74A or 3.1a, were used indiscriminately, no differences

being noted between them. Strain *nit-3A* (Fungal Stock no. 358; Fungal Genetics Stock Centre, California State University, Humboldt, Arkata, Calif. 95521, U.S.A.) lacks NADPH–nitrate reductase activity, is unable to use nitrate as a source of nitrogen, but retains a partial activity of nitrate reductase, i.e. Benzyl Viologen–nitrate reductase (Sorger, 1966).

Neurospora intermedia, *Neurospora tetrasperma* and *Neurospora sitophila* were from the collection of Professor S. F. H. Threlkeld, Department of Biology, McMaster University.

Cultures. The basic medium without a nitrogen source was described by Sorger & Giles (1965) and contained, in addition, one of the following nitrogen sources: ammonium tartrate, 4g/l ('ammonia medium'); $NaNO_3$, 5g/l ('nitrate medium'). Solid media contained, in addition, 1.5% agar (Difco).

The procedure for the intended separation of transcription and translation was a modified version of that described by Subramanian & Sorger (1972*b*). Mycelia were grown from a conidial inoculum into stationary culture for 40–44h at 27°C on ammonia medium, washed at least four times with water and transferred to basic medium with or without a nitrogen source and containing 0.5% (w/v) disodium EDTA and 1μg of cycloheximide/ml, usually for 2h at 27°C in shaking culture (step I). The mycelia were then washed at least four times with water and transferred to basic medium with no nitrogen source, containing 0.5% disodium EDTA, 1μg of cycloheximide/ml and 50μg of actinomycin D/ml for 2½h at 27°C in shaking culture (step II). The mycelia were then washed five times with water and transferred to basic medium with or without a nitrogen source, containing 0.5% disodium EDTA and 50μg of actinomycin D/ml, usually for 2½h at 27°C in shaking culture (step III). At the end of step III the mycelia were washed at least three times with water, blotted between paper towels until no more moisture was expressed and frozen in liquid N_2.

The presence of EDTA in the culture medium resulted in a lowering of the specific activity of nitrate reductase; however, this is compensated by the fact that actinomycin D penetrates the mycelia completely reproducibly as judged by its effect on synthesis of RNA and nitrate reductase.

When mycelia were to be incubated with radioactive isotopes the culture conditions were the same as described above. Mycelia were always harvested immediately after the incubation with radioactive material.

Methods

Extractions. Proteins were extracted as described previously (Subramanian & Sorger, 1972*b*) by grinding mycelial pads, which had been thawed from liquid N_2, with silica, with a mortar and pestle in ice-cold 0.1 M-potassium phosphate buffer, pH7.0 (2.0ml of buffer per mycelial pad of approx. 110mg blotted weight). The resulting brei was centrifuged at 12000*g* for 20min in a Servall refrigerated centrifuge and the supernatant was kept on ice until used, which was usually within 2h. The concentration of protein in such extracts was 2.0–5.4mg/ml.

Radioactive proteins were extracted as described above and samples (0.5ml) were withdrawn, precipitated with 2.0ml of 10% (w/v) trichloroacetic acid containing unlabelled 0.01 M-amino acid, incubated for 10min at room temperature and centrifuged at highest speed in a bench centrifuge for 10min. The supernatant was kept to count the radioactivity of acid-soluble material. The resulting pellet was then washed by repeating the acid-precipitation procedure two more times. This method succeeded in removing more than 99.94% of the free ^{14}C-labelled amino acids from an extract containing unlabelled protein and 1.25μCi of added [U-^{14}C]leucine (specific radioactivity 240μCi/mmol)/ml and 2.50μCi of added [U-^{14}C]phenylalanine (specific radioactivity 315μCi/mmol)/ml. The final pellet was resuspended in the above acid solution and collected on membrane filters (0.45μm pore size; Sartorius, Göttingen, Germany) with suction. The filters were then dried for 30–40min at 65°C and placed in toluene containing 4g of Omnifluor (New England Nuclear Corp., Boston, Mass., U.S.A.)/l, for radioactivity counting in a Beckman model LS-230 liquid-scintillation counter with an efficiency of counting of 45% for ^3H and 95% for ^{14}C.

A sample (0.5ml) of the supernatant from the first acid precipitation was added to 5.0ml of scintillation mixture prepared for aqueous samples (1 part of Triton X-100 to 2 parts of toluene, containing 4g of Omnifluor/l, final concn.) and its radioactivity counted.

RNA was extracted by two procedures.

Method 1 (Penman, 1966). Mycelia were withdrawn from liquid N_2 and ground with silica for a few seconds before addition of 2.0ml of 0.01 M-Tris–HCl buffer, pH7.5, containing 0.01 M-disodium EDTA, 0.10 M-NaCl and 2.0% (w/v) sodium dodecyl sulphate per mycelial pad and grinding for another 30s. The brei was added with vigorous agitation to an equal volume of water-saturated phenol and heated at 60°C with occasional agitation for 2min and subsequently cooled. The resulting mixture was then centrifuged and the aqueous layer plus the material at the interface was transferred to an equal volume of chloroform containing 1% (v/v) 3-methylbutan-1-ol and incubated with moderate agitation for 1h. The mixture was then centrifuged for 10min in a bench centrifuge at top speed and the non-aqueous layer removed, to be replaced by an equal volume of fresh chloroform–3-methylbutan-1-ol solution. The last

step was repeated three times. The aqueous supernatant was removed and incubated at 4°C overnight with 6ml of aq. 95% (v/v) ethanol. The precipitate was then redissolved in 2.0ml of 0.01M-Tris–HCl, pH7.5, containing 0.01M-disodium EDTA, 0.10M-NaCl and 0.5% sodium dodecyl sulphate. The E_{260} of this final solution was between 4.6 and 7.5 units. Samples (1.0ml) were removed, precipitated with 2.0ml of 10% trichloroacetic acid containing unlabelled 0.01M-uridine and centrifuged at top speed in a bench centrifuge. The pellet was resuspended in the same acid solution and the above procedure repeated twice. The final pellet was resuspended, placed on a membrane and the radioactivity counted as described for the protein preparations.

Method 2. Each deep-frozen mycelial pad was ground to a paste in a mortar and pestle on ice with silica and 0.20ml of a 1% (w/v) suspension of Macaloid in water. To this 2ml of 0.01M-Tris–HCl, pH7.5, containing 0.01M-disodium EDTA, 0.10M-NaCl and 2% sodium dodecyl sulphate was then added and the paste ground to a brei. The brei was then centrifuged at 12000*g* in a refrigerated centrifuge and the pellet discarded. A portion (1ml) of the supernatant, which had E_{260} between 26.0 and 54.0, was added to 2.0ml of 10% trichloroacetic acid and incubated for 10min before centrifuging at top speed in a bench centrifuge. The resulting supernatant was kept for counting the radioactivity of the acid-soluble material, and the pellet was resuspended and washed twice in 5% trichloroacetic acid containing unlabelled 0.01M-uracil. The final pellet was resuspended in the same 5% acid solution and the suspension divided on two membrane filters, each of which was then introduced into a separate scintillation vial and counted for radioactivity.

A portion (0.5ml) of the supernatant from the first acid precipitation was used to count radioactivity of acid-soluble material, as with the protein preparations.

The results obtained by the two methods were very similar.

Enzyme and protein assays. NADPH–nitrate reductase and Benzyl Viologen–nitrate reductase were assayed as described previously (Subramanian & Sorger, 1972*a*). One unit of activity is defined as the production of 1nmol of nitrite/min in the 0.5ml reaction mixture. Specific activity is expressed as units/mg of protein. When the specific activity of a preparation was below 7.0, the assay incubation time was doubled to obtain more reliable results. The specific activity of the preparation was not changed by this modification.

Xanthine dehydrogenase was measured by the procedure of Pateman *et al.* (1964).

Protein was measured by the biuret method (Dawson *et al.*, 1959) by using a protein-concentration reference curve made with bovine serum albumin.

Materials

Radioactive amino acids were from International Chemical and Nuclear, Irvine, Calif., U.S.A. Radioactive uridine was from Amersham–Searle Corp., Arlington Heights, Ill., U.S.A. Omnifluor was from New England Nuclear Corp., Boston, Mass., U.S.A. Actinomycin D was purchased in part from Sigma Chemical Co., St. Louis, Mo., U.S.A., and in part from Schwarz–Mann, Orangeburg, N.Y., U.S.A. A generous gift of actinomycin D was received from Merck, Sharp and Dohme (Canada) Ltd., Pointe Claire, Dorval, P.Q., Canada. Cycloheximide, NADPH, FAD, Triton X-100 and bovine serum albumin were from Sigma; Macaloid and Benzyl Viologen were from Schwarz–Mann. All other chemicals were reagent grade and were from Fisher Scientific Co., Fairlawn, N.J., U.S.A., or from Baker Chemical Co., Philipsburg, N.J., U.S.A.

Results

Nitrate is required for the induced synthesis of NADPH–nitrate reductase in the wild-type strain. Nitrate is not required for the de-repressed synthesis of Benzyl Viologen–nitrate reductase in strain *nit*-3, a mutant lacking NADPH–nitrate reductase, but retaining the related partial activity Benzyl Viologen–nitrate reductase. Ammonia represses the synthesis of the enzyme in both cases (Table 1).

The effect of various conditions on the transcription of nitrate reductase mRNA was examined by observing the influence of these conditions on the accumulation of the capacity by the mycelium to synthesize nitrate reductase in the presence of cycloheximide (see the Materials and Methods section, step I). Under such conditions general protein synthesis is virtually abolished, even though the uptake of leucine is not blocked (Fig. 1*b*). The cycloheximide-resistant synthesis of protein may be analogous to the situation described by Weiss *et al.* (1971) in *Neurospora*. These authors claimed that the cycloheximide-resistant protein synthesis was mitochondrial.

The presence of cycloheximide in step I does not seem to have a marked effect on general RNA synthesis or on the uptake of uracil (Figs. 2*a* and 2*b*). These results would suggest that nitrate reductase mRNA should accumulate under the right conditions during step I without being translated.

The effect of various conditions on the translation of nitrate reductase mRNA was examined by observing the influence of these conditions on the synthesis of nitrate reductase from preformed nitrate reductase mRNA that had presumably accumulated in step I. The ideal situation would have been to examine the translation of preformed RNA in the total absence of new RNA formation. The conditions of step III were the closest approximation to this situation obtainable in our hands.

Table 1. *Effect of different nitrogen sources on the induced synthesis of NADPH–nitrate reductase in the wild-type strain and of Benzyl Viologen–nitrate reductase in strain nit-3*

Mycelia were grown on ammonia medium, washed several times with water and transferred to basic medium containing 0.5% (w/v) disodium EDTA and the nitrogen sources shown, for 3½h in shaking culture at 27°C.

Strain	N source in 'induction medium'	Specific activity (nmol of nitrite produced/min per mg of protein)
Wild-type	No nitrogen	1.7, 1.3*
	20mM-$NaNO_3$	11.6, 11.8*
	20mM-$NaNO_3$+cycloheximide (1 μg/ml)	0.3
	10mM-Ammonium tartrate	0.9
nit-3	No nitrogen	90.5
	20mM-$NaNO_3$	127.8
	No nitrogen+cycloheximide (1 μg/ml)	1.6
	10mM-Ammonium tartrate	5.6, 4.1*

* Results from duplicate experiments.

Actinomycin D does not have its maximum effect immediately (Subramanian & Sorger, 1972*b*), and thus a preincubation stage (step II) followed step I. During step II protein synthesis was blocked with cycloheximide, and by the end of step II the presence of actinomycin D in the culture medium had a profound inhibitory effect on general RNA synthesis without seemingly blocking uracil uptake (Fig. 2*d*).

When the mycelia were transferred to fresh medium containing actinomycin D but no cycloheximide (step III), protein synthesis was resumed after 30–40min recovery from the treatment in step I and II (Fig. 1*c*). General RNA synthesis appears to be inhibited by about 50% in step III (Fig. 2*e*). The results shown in Figs. 2(*d*) and 2(*e*) would suggest that uracil uptake in the presence of actinomycin D is not limiting the biosynthesis of radioactive RNA in this case, as has been suggested by Tisdale & De Busk (1972) from their observations with *Neurospora* conidia.

If one assumes that a constant proportion of the RNA made in steps I, II and III is nitrate reductase mRNA, then one would expect that 50–60% of the total nitrate reductase mRNA made in steps I, II and III would have been made in steps II and III. The rationale for this calculation is that if 100 units of nitrate reductase mRNA are synthesized/h in step I, 25–50 units/h and 50 units/h would be synthesized in steps II and III respectively.

The maximum potential to synthesize nitrate reductase that is accumulated in steps II and III (0min in step I in Figs. 3 and 4, and no step I treatment in Table 2) is roughly one-third of the maximum potential to synthesize nitrate reductase that is accumulated in steps I, II and III (120min in step I in Figs. 3 and 4, and 120min step I treatment in Table 2). This is less than the 50–60% predicted on the basis of the weak assumption about the proportionality of mRNA to total RNA synthesis in the presence of cycloheximide and of actinomycin D. Given the variability of the system this agreement is encouraging.

The presence of nitrate in step I is not required for the accumulation of the potential to synthesize NADPH–nitrate reductase by the wild-type strain, or for the accumulation of the potential to synthesize Benzyl Viologen–nitrate reductase by strain *nit*-3 (Table 2 and Fig. 3). The presence of ammonia in step I seems to inhibit the accumulation of the capacity to synthesize nitrate reductase and components of this enzyme in both strains (Table 2 and Fig. 4).

The plots representing the accumulation of the potential to synthesize nitrate reductase in step I were variable. They were sometimes smooth upward curves, but more frequently were biphasic as shown in Fig. 3(*a*). Frequently, but not invariably, the accumulation of the capacity to synthesize nitrate reductase in step I was highest in the presence of nitrate for the first 20–30min, as shown in Fig. 3(*a*) and as observed previously (Subramanian & Sorger, 1972*b*). The rate of accumulation on no nitrogen source and on nitrate would sometimes become similar after the first 30min, but more frequently the rate of accumulation on nitrate would actually be less, as shown in Fig. 3(*b*).

The observations displayed in Figs. 3 and 4 and Table 2 are representative of a consistent pattern found in 19 comparable experiments with strain *nit*-3. These experiments differed in the length of incubation in steps I and III and in the final concentration of nitrogenous compound in the culture medium in step

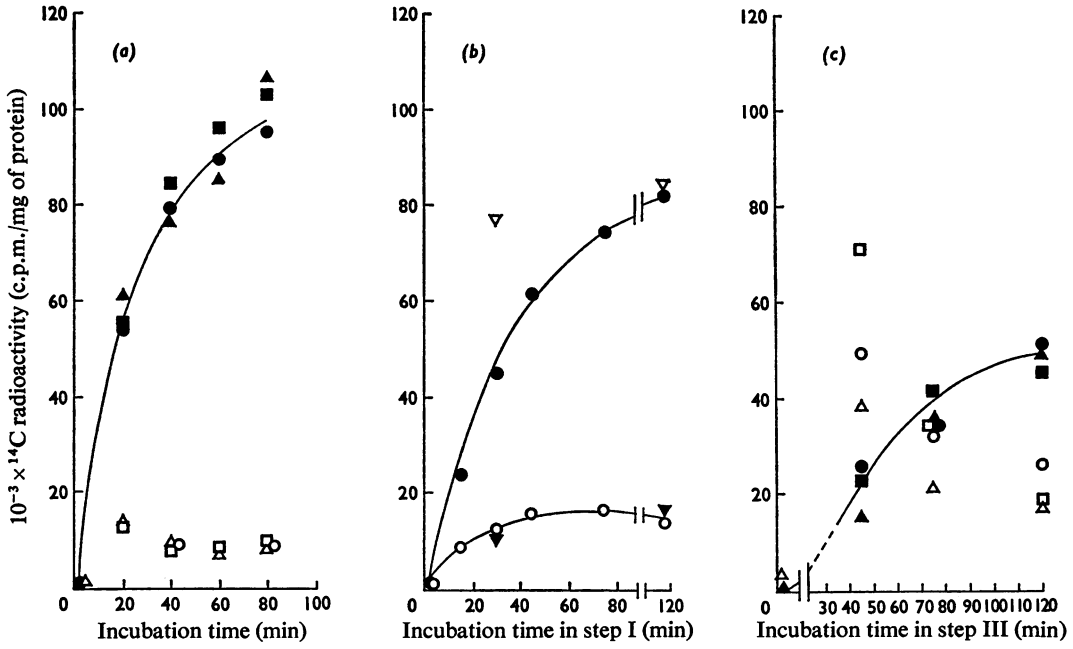

Fig. 1. *General protein synthesis and uptake of* [^{14}C]*leucine in* (*a*) *step I conditions in the absence of drugs and the presence of different nitrogen sources,* (*b*) *step I conditions in the presence* (▼, ▽) *or absence* (●, ○) *of* 1 μg *of cycloheximide/ml and without nitrogen source and* (*c*) *step III conditions in the presence of* 50 μg *of actinomycin D/ml and of different nitrogen sources*

(*a*) and (*c*): ▲, 10mM-NH_4^+ present; ■, 20mM-NO_3^- present; ●, no nitrogen source. (*a*)–(*c*): △, □, ○, ▽, Acid-soluble counts; ▲, ■, ●, ▼, acid-precipitable counts. Mycelial pads were grown into early stationary phase and washed with water. They were then incubated in step I conditions in the presence of 0.25 μCi of [^{14}C]leucine (311 mCi/mmol)/ml and of 1 μg of cycloheximide/ml, or in 'mock step I' conditions in the presence of label and the absence of drugs (*a*, *b*). In the experiment shown in (*c*), mycelial pads pregrown on ammonia medium were first given the usual step I and II treatments with drugs and no nitrogen source; they were then transferred to step III conditions in the presence of 50 μg of actinomycin D/ml, of the nitrogen source shown, and of 0.25 μCi of [^{14}C]leucine (311 mCi/mmol)/ml. At the desired time the pads were harvested, washed several times with water containing 1 μg of cycloheximide/ml, and quickly frozen in liquid N_2. The extraction and measurements of acid-precipitable and acid-soluble radioactivity and of protein were as described in the Materials and Methods section.

I. Frequent spot checks were made, and wild-type Benzyl Viologen–nitrate reductase behaved like NADPH–nitrate reductase.

The synthesis of the Benzyl Viologen–nitrate reductase of strain *nit*-3 begins somewhere between 30 and 60min incubation in step III in strain *nit*-3. In two separate experiments with the mutant, the specific activity of this enzyme was 0 at the beginning of step III, and, after 30min incubation in step III, 1.1 and 2.8 respectively at 60min, 4.4 at 90min and 10.6 and 14.0 at 120min. The wild-type strain behaved like strain *nit*-3 in this regard, NADPH–nitrate reductase activity appearing only after 30min incubation and before 60min incubation in step III (Subramanian & Sorger, 1972*b*; K. N. Subramanian & G. J. Sorger, unpublished work).

Nitrate stimulates the rate of accumulation of NADPH–nitrate reductase in step III at least threefold in the wild-type strain (Fig. 5*a*). In the absence of nitrate, there is little difference between the final accumulated specific activity of nitrate reductase in the presence or absence of ammonia (Fig. 6*a*). Almost identical results were obtained with the wild-type Benzyl Viologen–nitrate reductase (observations not shown). The initial rate of accumulation of Benzyl Viologen–nitrate reductase by strain *nit*-3 in step III was the same in the presence and absence of nitrate (Fig. 5*b*) as long as ammonia was absent. The final accumulated concentration of Benzyl Viologen–nitrate reductase in strain *nit*-3 preparations is 1.5 times higher when the mycelia were given the step III treatment in the presence of 20mM-nitrate than when

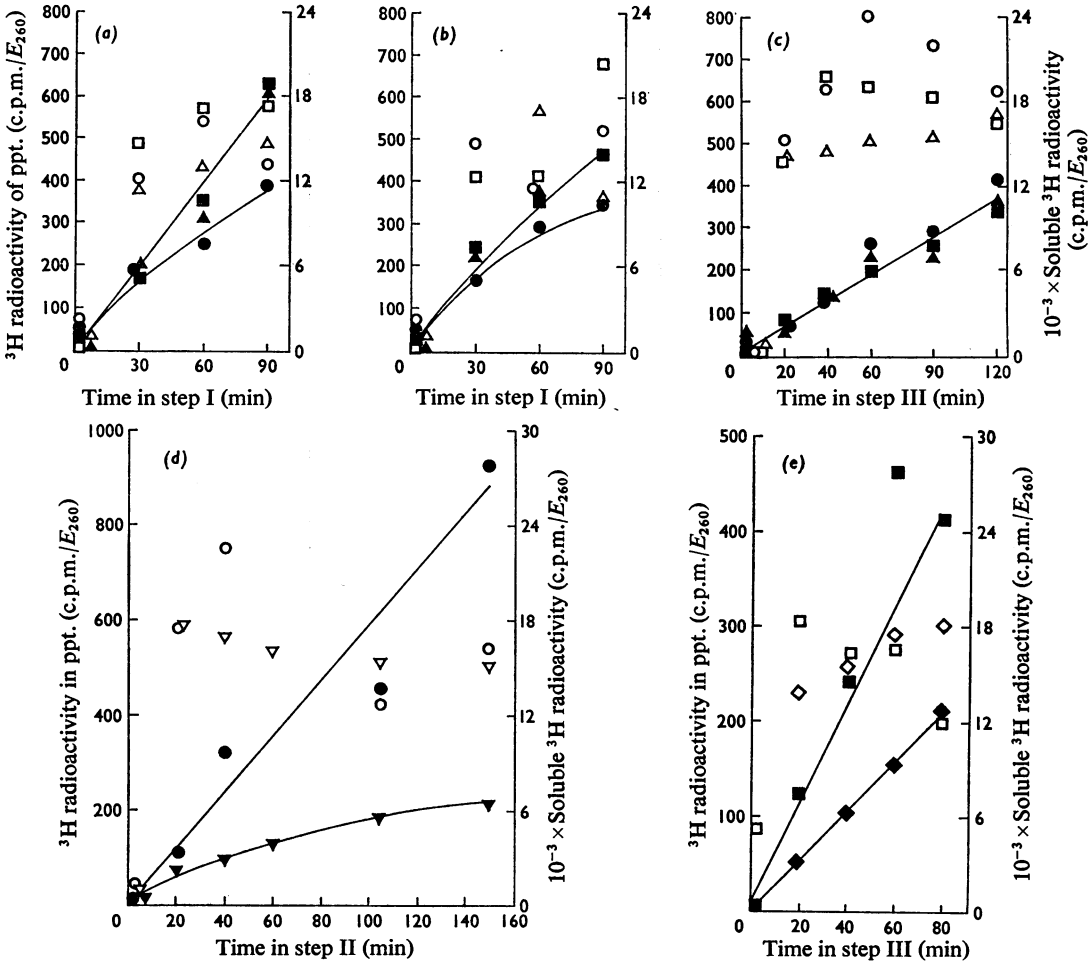

Fig. 2. *General RNA synthesis and uptake of [^{3}H]uridine under various conditions*

General RNA synthesis and uptake of [^{3}H]uridine were measured in (*a*) step I conditions in the absence of drugs and in the presence of different nitrogen sources, (*b*) step I conditions in the presence of cycloheximide and different nitrogen sources and (*c*) step III conditions in the presence of actinomycin D and different nitrogen sources. Nitrogen source: △, ▲, 10mM-NH_4^+ present; □, ■, 20mM-NO_3^- present; ●, ○, no nitrogen source. (*d*) The effect of the presence (▽, ▼) or absence (○, ●) of actinomycin D plus cycloheximide in step II conditions and no nitrogen source on general RNA synthesis and the uptake of [^{3}H]uridine. (*e*) The effect of the presence (◇, ♦) or absence (□, ■) of actinomycin D alone in step III conditions in the presence of 20mM-NO_3^- on general RNA synthesis and the uptake of [^{3}H]uridine. △, □, ○, ▽, ◇, Acid-soluble counts; ▲, ■, ●, ▼, ♦, acid-precipitable counts. Mycelia were grown into early stationary phase in ammonia medium and washed with water. They were then (*a*, *b*) incubated in step I conditions as described in the presence of [^{3}H]uridine, or (*c*) given the usual step I and step II treatments with no nitrogen source and with drugs before transfer to step III conditions in the presence of actinomycin D and [^{3}H]uridine. (*d*) and (*e*): Mycelial pads, pregrown into early stationary phase on ammonia medium, were washed and separated into two groups. For (*d*) one group received the usual step I and step II treatments with drugs and no nitrogen source (▼, ▽), and the other group was incubated under similar conditions but without drugs (●, ○). For (*e*) one group received the usual step I, II and III treatments with drugs (♦, ◇), and the other group was incubated under similar conditions but without drugs (■, □). In (*d*) and (*e*) radioactive uridine was present only during real or 'mock' step III. The radioactive medium contained in all cases 1 μCi of [^{3}H]uridine (25 Ci/mmol)/ml and was made 1 μM with respect to unlabelled uracil. At the desired time mycelia were harvested, washed with water, blotted dry and quickly frozen in N_2. The extraction and measurement of acid-soluble and acid-precipitable counts was as described in the Materials and Methods section.

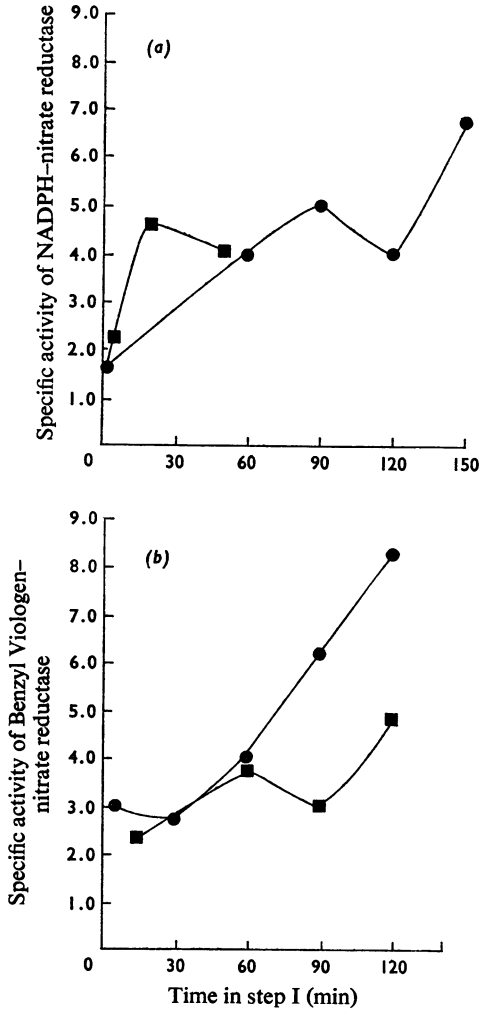

Fig. 3. *Accumulation of the potential to synthesize (a) NADPH–nitrate reductase during step I by the wild-type strain and (b) Benzyl Viologen–nitrate reductase by strain nit-3 in the presence or absence of nitrogen source*

Mycelia were pregrown on ammonia medium and given the step I treatment as shown, followed in all cases by a standard step II with no nitrogen source (●) and finally by step III in the presence of 20mM-$NaNO_3$ (■).

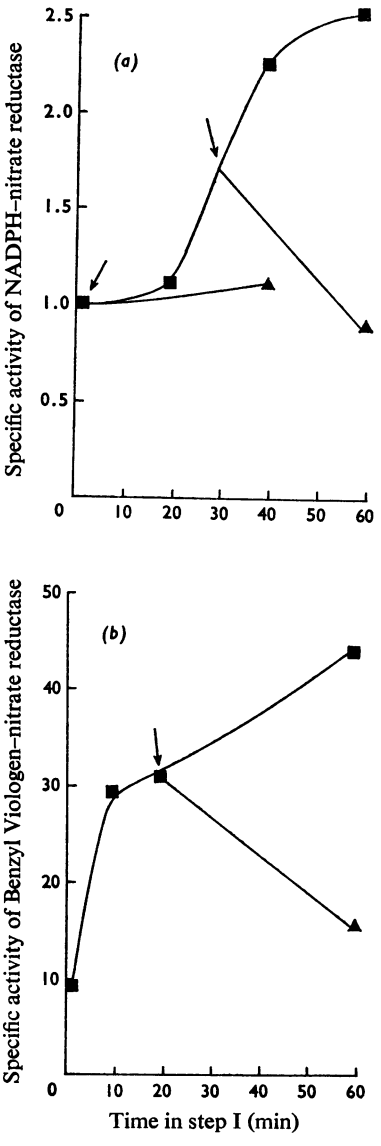

Fig. 4. *Effect of ammonium tartrate on the accumulation of the potential to synthesize (a) NADPH–nitrate reductase by the wild-type strain and (b) Benzyl Viologen–nitrate reductase by strain nit-3 during step I*

Mycelia were pregrown on ammonia medium and transferred to step I conditions in the presence of 2mM-$NaNO_3$ (■) or 10mM-ammonium tartrate (▲) for the designated times. The arrow denotes the time of addition of 10mM-ammonium tartrate to the step I incubation medium. Steps II and III were as described in the legend to Fig. 3.

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they were so treated in the absence of a nitrogen source (Fig. 6*b*). There is a big difference between the final specific activity of Benzyl Viologen–nitrate reductase accumulated in step III in the presence and absence of ammonia (Fig. 6*b*).

The presence of ammonia in step III stops the

Table 2. *Effect of different nitrogen sources in step I on the accumulation of the potential to synthesize nitrate reductase*

Mycelia were pregrown on ammonia medium, given the step I treatment for 2h or 0h with different nitrogen sources, followed by the standard step II and step III treatments, with no nitrogen source and with 20mM-$NaNO_3$ respectively. In the wild-type strain the specific activity ratio refers to NADPH–nitrate reductase and in strain *nit*-3 to Benzyl Viologen–nitrate reductase. Specific activity is as defined in Table 1.

Strain	N source in step I	No. of expts.	Ratio (specific activity after 120min of step I treatment/ specific activity with no step I treatment)
Wild-type	None	6	3.7±1.2
	20mM-$NaNO_3$	2	2.5, 2.8
	10mM-Ammonium tartrate	4	1.7±0.4
nit-3	None	2	2.8, 3.0
	20mM-$NaNO_3$	2	2.8, 1.7
	10mM-Ammonium tartrate	1	1.0

synthesis of NADPH–nitrate reductase in the wild-type strain and of Benzyl Viologen–nitrate reductase in strain *nit*-3 (Figs. 7*a* and 7*b*).

The results shown in Figs. 5, 6 and 7 are representative of a consistent pattern observed in ten comparable experiments with the wild-type strain and ten comparable experiments with strain *nit*-3. These experiments differed, however, in the length of incubation and the final concentration of nitrate in step I.

The effects of the presence of different nitrogen sources on the induction of nitrate reductase cannot be explained on the basis of their effects on general protein synthesis (Fig. 1*a*) or on general RNA synthesis (Fig. 2*a*).

The effects of the presence of the different nitrogen sources in step I on the accumulation of the capacity to synthesize NADPH–nitrate reductase and Benzyl Viologen–nitrate reductase do not seem to be due to effects on general RNA synthesis in step I (Fig. 2*b*).

The effects of the presence of different nitrogen sources in step III on the synthesis of nitrate reductase do not appear to be due to effects on general protein synthesis in step III (Fig. 1*c*) or to effects on actinomycin D-resistant RNA synthesis in step III (Fig. 2*c*).

The results shown in Fig. 1 are representative of those obtained from a second series of experiments using a mixture of [^{14}C]phenylalanine and [^{14}C]-leucine.

The results depicted in Fig. 2 were all obtained by using RNA-extraction method 2. The same pattern as shown in Fig. 2 was found by using RNA-extraction method 1.

The effect of no nitrogen source in step III on the net synthesis of NADPH–nitrate reductase by the wild-type strain is fully reversible by 20mM-nitrate, at least until 90min (Fig. 8).

The wild-type strain accumulated nitrate reductase activity in 20mM-nitrate in step III at a maximum rate of 0.054 unit of specific activity/min. The maximum rate of decline on transfer to no nitrogen source from these conditions was 0.046 unit of specific activity/min. At the time of transfer, the rate of synthesis of nitrate reductase on no nitrogen source in step III appeared to be zero (Fig. 9). In a separate experiment (not shown) with the wild-type strain, the maximum increase in 0.6mM-nitrate in step III was 0.038 unit of specific activity/min, the maximum decrease on transfer to no nitrogen source was 0.028 unit of specific activity/min and the rate of increase on no nitrogen source at the time of transfer was nil.

Neurospora tetrasperma appears to behave much like *Neurospora crassa*, requiring nitrate for induction and for step III synthesis of nitrate reductase, but not for the accumulation of the potential to make nitrate reductase in step I (Table 3).

Similar patterns, but with relatively high actinomycin D-resistant accumulation of nitrate reductase-synthesizing potential in steps II and III, were found with *Neurospora intermedia* and *Neurospora sitophila* (results not shown). Apparently the results obtained with *Neurospora crassa* 74A and 3.1a are not restricted to these two strains.

The observation that ammonia in step II causes an irreversible and considerable inhibition of the capacity to synthesize nitrate reductase in step III (Subramanian & Sorger, 1972*b*) was confirmed. The presence of ammonium tartrate in step II at a concentration of 3mM or greater resulted in the irreversible loss of the capacity to synthesize enzyme

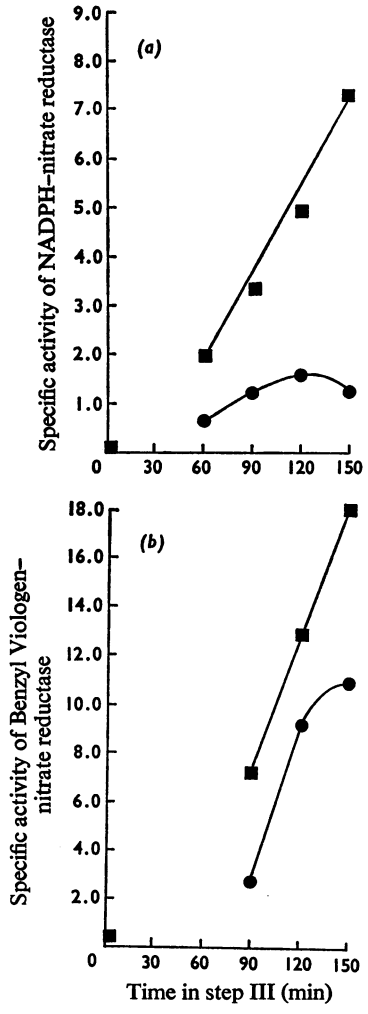

Fig. 5. *Effect of* 20mM-$NaNO_3$ *and of no nitrogen source in step III on the net synthesis of* (*a*) *NADPH–nitrate reductase by the wild-type strain and* (*b*) *Benzyl Viologen–nitrate reductase by strain nit*-3

Mycelia were pregrown on ammonia medium, given a 2h step I treatment with no nitrogen source, followed by the standard step II treatment and the step III treatment shown. ■, 20mM-$NaNO_3$ present; ●, no nitrogen source.

by the wild-type strain and by strain *nit*-3. Nitrate, on the other hand, had very little, if any, effect on step II (results of nine experiments, not shown).

Discussion

The results described above suggest that nitrate is not essential for the transcription of nitrate

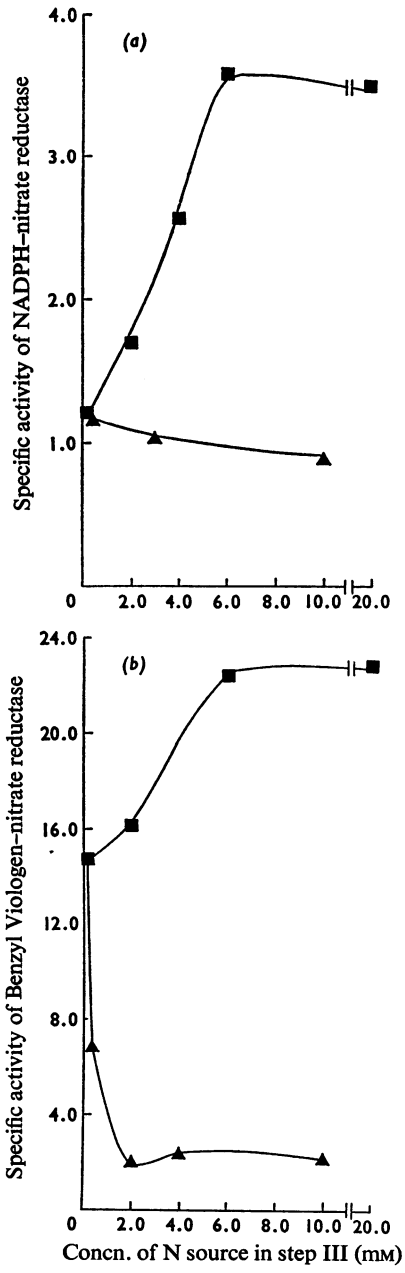

Fig. 6. *Effect of the concentration of sodium nitrate and of ammonium tartrate in step III on the net synthesis of* (*a*) *NADPH–nitrate reductase by the wild-type strain and* (*b*) *Benzyl Viologen–nitrate reductase by strain nit*-3

Mycelia were pregrown in ammonia, given 2h of step I treatment with no nitrogen source, followed by the standard step II treatment and 2½h of step III treatment in the presence of the nitrogen sources shown: ■, $NaNO_3$; ▲, ammonium tartrate.

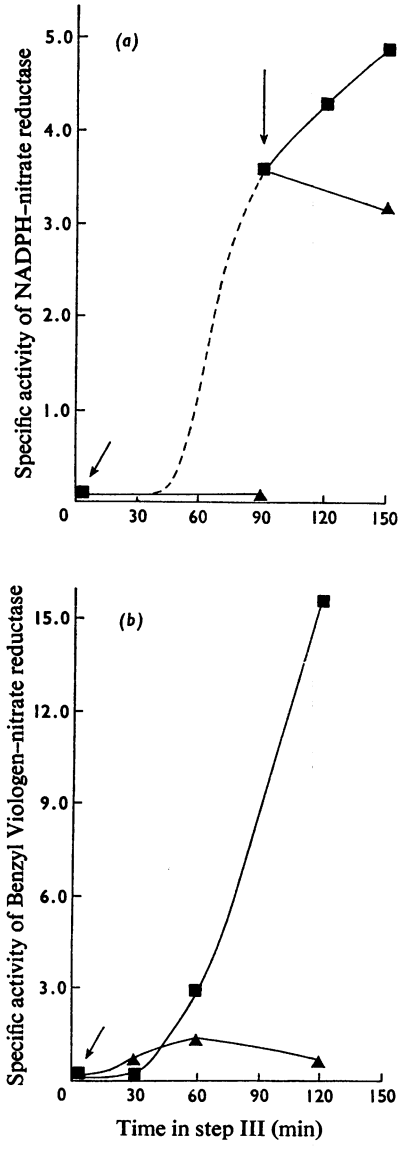

Fig. 7. *Effect of ammonia on the net synthesis of* (*a*) *NADPH–nitrate reductase by the wild-type strain and* (*b*) *Benzyl Viologen–nitrate reductase by strain nit*-3

Mycelia were pregrown in ammonia medium, given 30min of step I treatment in the presence of 2mM-$NaNO_3$, followed by the standard step II treatment and the step III treatment shown, in the presence of 20mM-$NaNO_3$ (■) or 20mM-$NaNO_3$ plus 10mM-ammonium tartrate (▲). The arrow indicates the time of addition of the ammonium tartrate.

reductase gene(s), but that it is important for the successful translation of the mRNA type(s) in the wild-type strain. The action of ammonia seems to be to antagonize successful transcription and to prevent successful translation, possibly by causing an accelerated inactivation or destruction of nitrate reductase mRNA species.

It is not possible at the moment to distinguish between two possible ways in which nitrate may affect the normal translation of nitrate reductase mRNA species: (*a*) the effector may influence the mechanism of translation itself, such as has been suggested by Tomkins *et al.* (1969) and by Levinson *et al.* (1971) to explain aspects of the regulation of tyrosine aminotransferase in mammalian systems, and by Artman & Ennis (1972) to explain aspects of the regulation of β-galactosidase in *Escherichia coli*; (*b*) nitrate may have an effect on the stability of the enzyme once it is formed, or as it is being formed, thus influencing the steady-state concentration of nitrate reductase by influencing its turnover, analogous to the hypothesis advanced by Schimke *et al.* (1965) to explain the regulation of tryptophan pyrrolase by tryptophan.

It is tempting, but not compelling, to propose possibility (*b*) because: (i) the rate of synthesis of wild-type nitrate reductase in the presence of nitrate in step III is not much greater than the rate of apparent destruction or inactivation in step III when nitrate is removed (see Fig. 8); (ii) the absence of a nitrogen source in step III has no permanent effect on the synthesis of nitrate reductase, as might be expected if mRNA were being destroyed or being converted irreversibly into an inactive form (see Fig. 9); (iii) the synthesis of Benzyl Viologen–nitrate reductase in strain *nit*-3 does not require the presence of nitrate in step III, and the Benzyl Viologen–nitrate reductase of this mutant seems to be relatively more stable than normal (Subramanian & Sorger, 1972*a*).

Cell-free extracts of the wild-type strain and of strain *nit*-3, grown for 2 days on ammonia medium and then incubated for 4h on hypoxanthine, had the same specific activity of xanthine dehydrogenase (G. J. Sorger, unpublished work). Strain *nit*-3 thus qualified, by analogy with the work of Cove (1970) and of Lewis & Fincham (1970*a*), as a likely mutant at one of the structural loci of nitrate reductase.

Nitrate reductase has been shown to be destroyed actively, presumably by proteolysis, in tobacco cells in tissue culture (Zielke & Filner, 1971). In the fungus *Ustilago maydis*, the ammonia-stimulated disappearance of this enzyme has been claimed to be due to degradation (Lewis & Fincham, 1970*b*). The prevention of protein synthesis slows down the rate of presumed destruction of nitrate reductase in barley (Travis *et al.*, 1969) and in *Ustilago* (Lewis & Fincham, 1970*b*), but not the ammonia-inactivation (without enzyme destruction) of nitrate reductase in

Table 3. *Effect of different nitrogen sources on the induction of nitrate reductase in Neurospora tetrasperma*

Mycelia were pregrown on ammonia medium: (*a*) subsequently they were treated as outlined in the legend of Table 1; (*b*) subsequently they were given the standard steps I, II and III treatments for 2, 2½ and 2½ h respectively with the N sources shown. Specific activity is as defined in Table 1.

(*a*) Induction without steps I, II and III

Treatment during 'induction'	N source in 'induction medium'	NADPH–nitrate reductase specific activity
3½h	20mM-$NaNO_3$	8.9
3½h+cycloheximide (1μg/ml)	20mM-$NaNO_3$	0.5
3½h	None	1.4
3½h	10mM-Ammonium tartrate	0.0

(*b*) Induction with steps I, II and III

N source			NADPH–nitrate reductase specific activity
Step I	Step II	Step III	
None	None	20mM-$NaNO_3$	2.2
None	None	None	0.2
20mM-$NaNO_3$	None	20mM-$NaNO_3$	2.6
20mM-$NaNO_3$	None	None	1.0
10mM-Ammonium tartrate	None	20mM-$NaNO_3$	1.4
10mM-Ammonium tartrate	None	None	0.0
No incubation	None	20mM-$NaNO_3$	1.3

Cyanidium caldarium (Rigano, 1971). The disappearance of nitrate reductase in *Neurospora* resembles the situation in higher plants and in *Ustilago* more than that in algae in this respect (Subramanian & Sorger, 1972*a*), suggesting that nitrate reductase is destroyed.

It is not difficult to envisage the destruction of nitrate reductase being responsive to structural or conformation changes of this enzyme. If a protease acts only on available peptide bonds, and has the specificity of action of chymotrypsin (Blow & Steitz, 1970), for example, any conformational change of nitrate reductase that would affect the availability of peptide bonds which can serve as protease substrates would automatically also affect the rate of turnover of nitrate reductase. The substrate nitrate and the effect of the mutation at the *nit*-3 locus could both result in the stabilization of nitrate reductase proteins to proteolytic destruction by affecting the conformation of the enzyme.

Cove & Pateman (1969) have postulated a model in which the nitrate reductase apoprotein of *Aspergillus nidulans* serves as an effector in its own synthesis. These authors propose that, in the presence of nitrate, nitrate reductase prevents the inducer protein from becoming a repressor protein, the result of which is the induction of nitrate reductase. The observations reported in the present paper would not seem to support Cove's (1970) model because nitrate is not required for the accumulation of what appears to be nitrate reductase mRNA.

Warner *et al.* (1969) recognized the importance of enzyme degradation in the regulation of nitrate reductase from corn. They observed that inbred lines of corn which had different rates of nitrate reductase accumulation during the induction of this enzyme had different rates of decay *in vivo* and also *in vitro*. It seems that the favoured explanation offered here to account for the results with *Neurospora* might be applicable to the results obtained with corn.

A consistent feature of the models proposed by Cove (1970) is that a mutation in the nitrate reductase 'structural locus' affects the regulation of both nitrate reductase and nitrite reductase, because it is proposed that the nitrate reductase apoprotein regulates the synthesis of both enzymes. Nitrite reductase does indeed seem to be constitutive in mutants of *Aspergillus nidulans* altered in the presumed 'structural gene' for nitrate reductase (Cove, 1970). The favoured explanation given in the present paper to explain the results with *Neurospora* would not account for such observations in *Aspergillus*, and could not account for the effect of ammonia

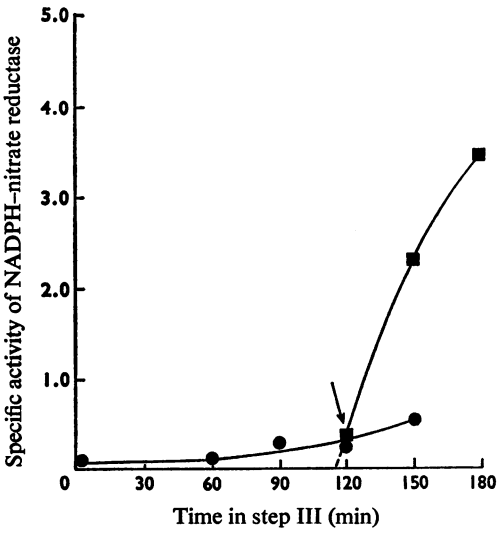

Fig. 8. *Reversal by 20mM-$NaNO_3$ of the effect of no nitrogen source, in step III, on the net synthesis of NADPH–nitrate reductase by the wild-type strain*

Mycelia were pregrown on ammonia medium, given step I treatment without a nitrogen source for 2h, followed by the standard step II treatment and the step III treatment shown above. The arrow indicates the time of addition of 20mM-$NaNO_3$ to some of the cultures (■); other cultures received no addition (●).

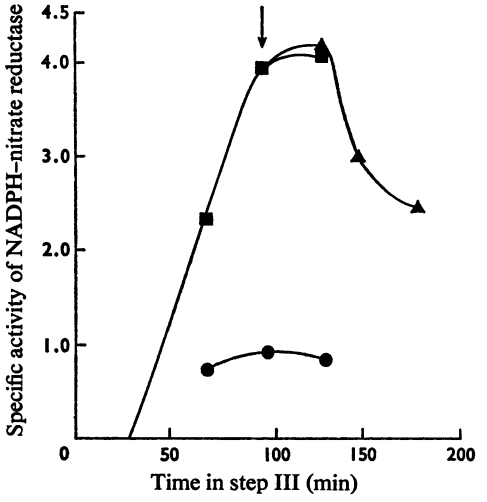

Fig. 9. *Effect of the removal of nitrate during step III on the net synthesis of NADPH–nitrate reductase by the wild-type strain*

Mycelia were pregrown on ammonia medium and given the same step I and II treatment as described in the legend to Fig. 8. Some of the cultures were incubated in step III in the presence of 20mM-$NaNO_3$ (■); other cultures were incubated without a nitrogen source (●). Some of the cultures incubated in step III with 20mM-$NaNO_3$ (■) were washed and transferred at the time shown by the arrow to fresh step III conditions in the absence of a nitrogen source (▲).

on transcription in *Neurospora*. Obviously there is more to the regulation of nitrate reductase than the regulation of the degradation of this enzyme.

The action of ammonia on transcription may be, but need not be, as a conventional co-repressor as conceived by Jacob & Monod (1961) in their model of end-product repression. It could act as an effector on a sigma-like subunit of RNA polymerase (Travers, 1971) and/or on a more generalized transcription effector such as the cyclic AMP–receptor protein described by de Crombrugghe *et al.* (1971), and/or its presence could result in the enhanced degradation or inactivation of nitrate reductase mRNA.

The last explanation seems particularly attractive because of the results shown in Fig. 4, because of the irreversible effect of the presence of ammonia in step II on the potential for nitrate reductase synthesis (see the Results section) and because of the effect of ammonia on the synthesis of nitrate reductase and related activities in step III in both the wild-type strain and strain *nit*-3 (Fig. 7).

In addition to its effect on the transcription of nitrate reductase genes or on mRNA stability or both, ammonia may have an effect on the translation mechanism of nitrate reductase mRNA and/or on the rate of degradation of nitrate reductase protein, but the results in the present paper do not support or reject such possibilities.

This investigation was supported by National Research Council of Canada Grant no. A3649. We are grateful to Dr. W. D. Dorian of Merck, Sharpe and Dohme (Canada) Ltd. for providing a generous gift of actinomycin D.

References

Artman, M. & Ennis, H. L. (1972) *J. Bacteriol.* **110**, 652–660

Bayley, J. M., King, J. & Gamborg, O. L. (1972*a*) *Planta* **105**, 15–24

Bayley, J. M., King, J. & Gamborg, O. L. (1972*b*) *Planta* **105**, 25–32

Blow, D. M. & Steitz, T. A. (1970) *Annu. Rev. Biochem.* **39**, 63–95

Cove, D. J. (1966) *Biochim. Biophys. Acta* **113**, 51–56

Cove, D. J. (1970) *Proc. Roy. Soc. Ser. B* **176**, 267–275

Cove, D. J. & Pateman, J. A. (1969) *J. Bacteriol.* **97**, 1374–1378

Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1959) *Data for Biochemical Research*, p. 84, Clarendon Press, Oxford
de Crombrugghe, B., Chen, D., Gottesman, M., Pastan, I., Varmus, H. E., Emmer, M. & Perlman, R. L. (1971) *Nature (London) New Biol.* **230**, 37–40
Evans, H. J. & Nason, A. (1953) *Plant Physiol.* **28**, 233–254
Filner, P. (1966) *Biochim. Biophys. Acta* **118**, 299–310
Hewitt, E. J., Notton, B. A. & Afridi, M. M. R. K. (1967) *Plant Cell Physiol.* **8**, 385–397
Ingle, J. (1968) *Biochem. J.* **108**, 715–724
Ingle, J., Joy, K. W. & Hageman, R. H. (1966) *Biochem. J.* **100**, 577–588
Jacob, F. & Monod, J. (1961) *J. Mol. Biol.* **3**, 318–356
Joy, K. W. (1969) *Plant Physiol.* **44**, 849–853
Kinsky, S. C. (1961) *J. Bacteriol.* **82**, 898–904
Levinson, B. B., Tomkins, G. M. & Stellwagen, R. H. (1971) *J. Biol. Chem.* **246**, 6297–6302
Lewis, C. M. & Fincham, J. R. S. (1970*a*) *Genet. Res.* **16**, 151–163
Lewis, C. M. & Fincham, J. R. S. (1970*b*) *J. Bacteriol.* **103**, 55–61
Losada, M., Paneque, A., Aparicio, P. J., Vega, J., Cárdenas, J. & Herrera, J. (1970) *Biochem. Biophys. Res. Commun.* **38**, 1009–1015
Morton, A. G. (1956) *J. Exp. Bot.* **7**, 97–112
Pateman, J. A., Cove, D. J., Rever, B. M. & Roberts, D. B. (1964) *Nature (London)* **201**, 58–60
Penman, S. (1966) *J. Mol. Biol.* **17** 117–130
Pichinoty, F. & Méténier, G. (1967) *Ann. Inst. Pasteur Paris* **112**, 701–711
Rigano, C. (1971) *Arch. Mikrobiol.* **76**, 265–276
Schimke, R. T., Sweeney, E. W. & Berlin, C. M. (1965) *J. Biol. Chem.* **240**, 322–331
Schrader, L. E., Ritenour, G. L., Eilrich, G. L. & Hageman, R. H. (1968) *Plant Physiol.* **43**, 930–940
Silver, W. S. (1957) *J. Bacteriol.* **73**, 241–246
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
Smith, F. W. & Thompson, J. F. (1971*a*) *Plant Physiol.* **48**, 219–223
Smith, F. W. & Thompson, J. F. (1971*b*) *Plant Physiol.* **48**, 224–227
Sorger, G. J. (1965) *Biochim. Biophys. Acta* **99**, 234–245
Sorger, G. J. (1966) *Biochim. Biophys. Acta* **118**, 484–494
Sorger, G. J. & Giles, N. H. (1965) *Genetics* **52**, 777–788
Stewart, G. R. (1968) *Phytochemistry* **7**, 1139–1142
Subramanian, K. N. & Sorger, G. J. (1972*a*) *J. Bacteriol.* **110**, 538–546
Subramanian, K. N. & Sorger, G. J. (1972*b*) *J. Bacteriol.* **110**, 547–553
Thacker, A. & Syrett, P. J. (1972) *New Phytol.* **71**, 435–441
Tisdale, J. H. & De Busk, A. G. (1972) *Biochem. Biophys. Res. Commun.* **48**, 816–822
Tomkins, G. M., Gelehrter, T. D., Martin, D., Jr., Samuels, H. H. & Brad-Thomson, E. (1969) *Science* **166**, 1474–1480
Travers, A. (1971) *Nature (London) New Biol.* **229**, 69–74
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
Warner, R. L., Hageman, R. H., Dudley, J. W. & Lambert, R. J. (1969) *Proc. Nat. Acad. Sci. U.S.* **62**, 785–792
Weiss, H., Sebald, W. & Bucher, T. (1971) *Eur. J. Biochem.* **22**, 19–26
Wray, J. L. & Filner, P. (1970) *Biochem. J.* **119**, 715–725
Zielke, R. H. & Filner, P. (1971) *J. Biol. Chem.* **146**, 1772–1779
Zumft, W. G., Spiller, H. & Yeboah-Smith, I. (1972) *Planta* **102**, 228–236