Running Title: Regulation of nitrite reductase in *Neurospora crassa*.

STUDY OF THE REGULATION OF NITRITE REDUCTASE IN *NEUROSPORA CRASSA*

By

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ABSTRACT

Two nitrite reductase activities were identified in crude extracts of wild type *Neurospora* exposed to nitrate ions: a first one catalyzes the stoichiometric reduction of nitrite to ammonia (AP); a second causes the disappearance of nitrite to something other than ammonia (ND). The second activity (ND) which is present in all strains under all conditions, was isolated by ultrafiltration through a collodion membrane or by precipitation with 50% acetone. This activity could account for the difference between the nitrite reduction and ammonia production catalyzed by crude extracts of fully induced wild type mycelia. The properties of the ND component were studied by examining the effect of organic solvents, proteases, sulfhydryl reagents, metal-complexing agents and temperature on its activity. The AP activity which is inducible by nitrate or nitrite and is present only in nitrite-utilizing strains, was deduced from a comparison of nitrite non-utilizing mutants and the wild type strain to be the assimilatory enzyme.

Nitrate was found to induce the assimilatory nitrite reductase directly. Four different approaches were employed to show this: a study of the kinetics of induction

of nitrate - and nitrite reductase; an examination of the induction of nitrite reductase by nitrate in the presence of tungstate or in the presence of ammonium ions; a comparison of the induction of nitrite reductase by nitrate in nitrate reductaseless mutants and in the wild type strain. Active nitrate reductase does not appear to be necessary for the induction of nitrite reductase by nitrate as suggested by Cove and Pateman (7). The nitrite reductase apoprotein, on the other hand, seems to be involved in the induction of the assimilatory nitrite reductase.

Ammonium ions repressed the induction of the assimilatory nitrite reductase by nitrate. This repressive effect was an indirect one; ammonium ions had to be metabolized first in order to repress nitrite reductase. This was shown by the lack of repression in strain *am-1a*, a glutamate dehydrogenaseless strain.

A model is proposed to describe the possible mechanism of induction and repression of nitrite reductase in *Neurospora crassa*.

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INTRODUCTION

With the ever increasing demand for food, there is an urgent need to increase the world's production of crops. To accomplish this, scientists have been working on the breeding of new varieties of crops which would be high-yielding, disease resistant, and highly responsive to fertilizers. One product of this research is the wide-spread use of "miracle" strains of wheat and rice which were the basis of high hopes and of the so-called "green revolution" in the decade 1960-1970. Because of their great responsiveness to nitrogen fertilizer, the uptake and metabolism of nitrate by the "miracle" strains is of great economic and social importance.

Of the four basic elements constituting the organic matter of a plant, carbon and oxygen are readily available from the air. Hydrogen is provided by water, which is available from irrigation or precipitation; and nitrogen is often limiting. Although nitrogen constitutes 80% of the atmosphere, most plants cannot utilize molecular nitrogen directly. Nitrogen is usually taken up by the plant root system in the form of nitrate, ammonia or urea from the soil. Leaves can also take up some of these nitrogenous compounds from the air or from sprayings.

Most soils contain nitrogenous compounds in varying amounts, but frequently there is not enough to support a good crop. Additional nitrogen must be supplied. This is usually done in two ways.

1. Use of nitrogen-fixing bacteria. Some bacteria are able to assimilate molecular nitrogen from the atmosphere to produce ammonia. One of the best known examples is the symbiotic nitrogen-fixing bacteria in the nodules of legumes. Henzell and Norris (20) reported that in warm northern New Zealand, about 400-500 pounds of nitrogen per acre are fixed by these bacteria symbiotically with white clover in one season. This amount of fixed nitrogen not only supplies the nitrogen needs of the existing crop, but can also supply the need of the next crop. This is one of the reasons why farmers rotate their crops with legumes.

2. Use of fertilizers. Manures and decomposed plant materials have long been used to restore nitrogen, phosphorus and potassium to the soil. In addition, naturally occurring forms of nitrate, mainly sodium and calcium nitrate are applied to the soil in many parts of the world. Since World War II, with the increases in crop production, artificial nitrogen fertilizers have become increasingly important. Today, most nitrogen fertilizers used are manufactured, mainly in the form of ammonia, nitrate and urea.

When ammonia is applied to the soil, it is often converted into nitrate by nitrifying bacteria present in the soil. The process can be very rapid during warm growing seasons, especially when the temperature exceeds 70 F. Hence, most if not all of the ammonia applied during the growing season may be converted to nitrate. Nitrate is thus the most important form of nitrogen assimilated by plants.

Two key enzymes involved in the assimilation of nitrate are: nitrate reductase, which reduces nitrate to nitrite, and nitrite reductase, which reduces nitrite to ammonia. The study of the regulation of these two enzymes has significant implications for the regulation of the nitrogen nutrition of plant crops.

Another point worth mentioning here is that with the increasing demand for food and the limitation of arable land, there will be an increasing dependence of human beings on plant proteins. It would thus seem necessary to improve the protein contents of the crops - both in quantity and in quality. An understanding of the regulation and mechanism of action of enzymes involved in the assimilation of nitrate would help to control the production of edible nitrogen by crops.

In this study, the regulation of nitrite reductase in *Neurospora crassa* was investigated. It is my hope that the information arising from this study can be applied to the cultivation of food crops.

A. General Description of the Enzyme

Nitrite reductase was first found in a cell-free extract of *Bacillus pyocyaneus* by Yamagata in 1939 (as cited in 42). Since then, the enzyme has been studied in plants, algae, fungi and microorganisms.

Nitrite reductase is an iron-protein. This was shown by the incorporation of ^{59}Fe into highly purified marrow (26) and *Chlorella* (1) nitrite reductase. Each enzyme molecule may contain two atoms of iron (4,26,75). The molecular weight of nitrite reductase has been studied in spinach (23), marrow (24), *Anabaena* (18), *Chlorella* (75), *Neurospora* (33), *Azotobacter* (71) and *Achromobacter* (48) by gel exclusion or sedimentation. It has been found to be between 61,000 and 70,000 except for the *Neurospora* enzyme (M.W. 290,000) and the *Achromobacter* enzyme (M.W. 95,000).

An inorganic pathway for the reduction of nitrite to ammonia was suggested by Nason in 1962 (42).

nitrite	→	hyponitrite	→	hydroxylamine	→	ammonia
NO_2^-		$H_2N_2O_2$		NH_2OH		NH_3

This pathway would require the transfer of six electrons to nitrite. This is supported by the observations that three molecules of NADH are oxidized for each molecule of nitrite reduced (30,41) and that six molecules of reduced benzyl viologen are oxidized for each molecule

of nitrite reduced (48). NADH is a two electron carrier and benzyl viologen is a single electron carrier.

In most assays, reduced ferredoxin (for plant systems, 21a), reduced pyridine nucleotides or reduced benzyl (methyl) viologen are used as the electron donors, and the oxidation of pyridine nucleotides or the disappearance of nitrite is measured as the enzyme activity. The production of ammonia is not always determined.

B. Lack of Stoichiometry Between Nitrite Reduction and Ammonia Production

Stoichiometric production of ammonia from the catalytic reduction of nitrite has been shown in many systems (8,18,25,30,33,48,71,75), however, recovery of less than 85% of the nitrite nitrogen in the form of ammonia has been reported in tomato (51), cultured tobacco cells (29), and diatoms (13) (Table 1). When extracts of soybean leaves (49) or of excised tomato roots (69) were used to catalyze the reduction of nitrite, no ammonia was detected. Nitrite was reduced non-stoichiometrically to ammonia by crude extracts of *Neurospora crassa* (40). Lafferty and Garrett were able to show stoichiometric production of ammonia from the reduction of nitrite with a 90-fold purified nitrite reductase from *Neurospora* (33).

Table 1

Summary of Studies on the Relationship between Nitrite Reduction and Ammonia Production

Organism	Electron donor in assay	Purity of enzyme	% of NO_2^- reduced recovered as NH_3	Reference No.
1. *E. coli*	NADH	crude extract	100	30
2. *Azotobacter*	NADPH + FAD	3 X purified	86 - 98	62
	NADH	crude extract	92 - 116	71
3. *Achromobacter*	B.V. + hydrosulfite	83 X purified	~95	48
	NADH + FMN	83 X purified	~100	48
4. *Neurospora*	B.V. + hydrosulfite	crude extract	50 - 80	40
	NADPH	90 X purified enzyme	100	33
5. *Ditylum*	M.V. + hydrosulfite	crude extract	~80	13
6. *Anabaena*	M.V. + hydrosulfite	40 X purified	90 - 120	18
7. *Chlorella*	NADPH	420 X purified	100	75
8. Tomato root		crude extract	0	69
9. Tomato plant	B.V. red	crude extract	~100	51
	NADPH + B.V.	crude extract	~80	51
10. Cultured tobacco cells	M.V. + hydrosulfite	crude extract	~85	29
11. *Cucurbita pepo*	B.V. red	crude extract	90 - 100	8
12. Corn scuttellum	M.V. + hydrosulfite	semi-purified	96 - 115	25
13. Maize, spinach	B.V. + hydrosulfite	29 X purified	85	28
	NADPH-GPD	66 X purified	75 - 108	28
14. Spinach	Isolated chloroplast + light		~90	39
15. Citrus leaves	B.V. + hydrosulfite	crude extract	~92	57
16. Soybean leaves	NADH	crude extract	0	49

Abbreviations

NADH Reduced nicotinamide adenine dinucleotide.
NADPH Reduced nicotinamide adenine dinucleotide phosphate.
FAD Flavin adenine dinucleotide.
B.V. Benzyl viologen.
FMN Flavin mononucleotide.
M.V. Methyl viologen.
GPD Glucose-6-phosphate dehydrogenase.
B.V. red Benzyl viologen partially reduced by hydrogen and palladized asbestos.

1. Complications in the nitrite reductase assay. The stoichiometry observed in assay systems where reduced pyridine nucleotides were used as the electron donors may be a false one, due to the following complications: Ammonia is produced non-enzymatically from the degradation of pyridine nucleotides (NAD) under the alkali micro-diffusion conditions employed for the detection of ammonia, and the oxidized form of the pyridine nucleotides (NAD^+) is more labile to this type of degradation than the reduced form (NADH) (51). Recently, Lafferty and Garrett (33) reported that only negligible degradation of the oxidized NADP was observed under the alkali conditions of micro-diffusion; possibly $NADP^+$ is more resistant to alkaline degradation than NAD^+.

Nitrite is measured colorimetrically by reacting it with sulfanilamide and naphthylethylene diamine dihydrochloride. Reduced pyridine nucleotides have been shown to interfere with the diazotization of nitrite with the color reagents used in its determination (37). For this reason, barium or zinc acetate, which precipitates the nucleotides, is used to terminate the assay before adding the color reagents (37). The above treatment was not effective in removing NADH from assay mixtures containing radish cotyledon extracts, and the use of activated charcoal was proposed for full recovery of nitrite in the assay (65). In some plant systems, malic

dehydrogenase and oxaloacetate were used to oxidize the residual NADH (21,63).

Another complication has been reported when purified enzyme was used to catalyze reduced benzyl viologen-nitrite reduction (22). Residual ammonium sulphate which may be present in the purified enzyme, even after dialysis or filtration of the enzyme through Sephadex-G25, would cause the loss of nitrite when reduced benzyl viologen was present in the assay mixture. It was reported that 0.36×10^{-3}M ammonium sulphate caused a loss of 620 nmoles of nitrite in 25 minutes under such conditions. This concentration of ammonium sulphate would correspond to the conditions achieved by the residual presence of about 1% of the ammonium sulphate required to precipitate the protein fraction containing the enzyme.

2. Presence of more than one species of nitrite reductase. One explanation for the lack of stoichiometry between the reduction of nitrite and the production of ammonia may be that there is more than one activity reducing nitrite in the enzyme preparation. In *Neurospora*, Mulkins proposed the presence of two nitrite reducing activities - one reducing nitrite to ammonia, the other reducing nitrite to something other than ammonia (40).

Hucklesby *et al.* (25) reported the existence of two forms of nitrite reductase in extracts of corn scutellum, which could be separated by DEAE-cellulose

column chromatography. The two forms showed a marked difference in thermal stability, ionic charge, and behaviour during iso-electric focusing, but shared many properties including molecular weight, Km for nitrite, pH optimum and stoichiometric production of ammonia from nitrite. Later, the two forms of nitrite reductase were shown to be present in the roots and etiolated shoots of corn whereas only one form was found in the corn leaves (9). Similar findings were reported from experiments with *Chlorella*: two peaks of nitrite reductase activity were separated by polyacrylamide gel electrophoresis of a partially purified enzyme preparation (75). The kinetics of decay of nitrite reductase *in vivo* in cultured tobacco cells suggests the presence of two species of nitrite reductase with different stabilities (29).

3. Products of nitrite reduction. Ammonia is widely accepted as the product of the enzymatic reduction of nitrite by nitrite reductase, yet the existence of other products of nitrite reduction has not been ruled out. Nason proposed an inorganic pathway for the reduction of nitrite to ammonia in which hyponitrite and hydroxylamine were intermediates (42). It has been shown that *E. coli* strain B/r can utilize nitrous oxide, hyponitrite, or hydroxylamine as sole nitrogen sources (35), but these compounds are not detectable as free intermediates in

cultures of *E. coli* grown on nitrate or nitrite. Enzymatic reduction of hyponitrite and hydroxylamine has been demonstrated by extracts of *Neurospora* (36). However, studies of nitrite and hydroxylamine reduction by extracts of *Cucurbita pepo* (8) showed that the Km of the preparation for hydroxylamine was higher than that for nitrite, suggesting that the presence of free hydroxylamine was unlikely. A similar difference in Km of nitrite reductase and hydroxylamine reductase for the above substrates has been observed in studies with *E. coli* (30). Free hydroxylamine is highly toxic, making it even less likely to be a free intermediate (73). The presence of other products is yet an open question. Enzyme-bound or compartmentalized intermediates have not been ruled out by the above considerations.

A nitrite reductase activity which does not catalyze the production of ammonia has been reported in extracts of citrus leaves, of excised tomato roots, and of other plant tissues (2,57,69). In all these cases, it was suggested that the disappearance of nitrite was caused by ascorbic acid.

It would seem from the above considerations, that the lack of stoichiometry observed between the reduction of nitrite and the production of ammonia catalyzed by enzymes can arise from artifacts of the assay, interfering

molecules in the extracts, or alternative pathways of reduction. If one assumes that the product of assimilatory nitrite reduction is ammonia, assays which only measure nitrite disappearance or oxidation of pyridine nucleotides may not be an accurate measurement of the assimilatory enzyme.

C. Regulation of Nitrite Reductase

1. Nitrate as the inducer of nitrite reductase. Nitrite reductase is an adaptive enzyme which is induced by its substrate nitrite. In most systems, the enzyme is also inducible by nitrate (Table 2). However, the role of nitrate in induction differs from one system to another. Nitrate was believed to induce nitrite reductase directly in *Azotobacter* (71), *Aspergillus* (47), *Neurospora* (16), *Lemna minor* (64), *Agrostemma* embryos (11) and cultured tobacco cells (29) (Table 2) because of one or two of the following observations:

1. Nitrite and nitrate reductases were induced simultaneously by nitrate.

2. Normal nitrite reductase activity was formed when tungstate was present in the nitrate induction medium. Tungstate is reported to prevent the formation of active

Table 2

Summary of Studies on the Regulation of Nitrite Reductase

Organism	Inducer	Role of NO_3^- as inducer (evidence*)	Synthesis of enzyme inhibited by	Induction repressed by	Reference No.
1. *E. coli*	NO_2^-			NH_4^+ (slightly)	30
2. *Azotobacter*	NO_3^-; NO_2^-	direct (tungstate)		NH_4^+ in NO_3^- medium	71
3. *Aspergillus*	NO_3^-; NO_2^-	direct (NR^- mutant)		NH_4NO_3	48
4. *Neurospora*	NO_3^-; NO_2^-	direct (kinetics)		NH_4NO_3; casamino acids	16 56
5. *Ditylum*	NO_3^-; NO_2^-			NH_4^+ (slightly)	13
6. *Anabaena*	NO_3^-; NO_2^-	indirect (kinetics)			44
7. *Chlorella*	NO_2^-		Actinomycin D		32
	NO_3^-	derepression	Cycloheximide	NH_4^+	34
8. *Lemna*	NO_3^-; NO_2^-	direct (kinetics, tungstate)	Cycloheximide, Chloroamphenicol	NH_4^+; CA AAs	64
9. Radish cotyledons	NO_3^-; NO_2^-	indirect (kinetics)	Actinomycin D, puromycin		27
10. *Agrostemma*	NO_3^-; NO_2^-	direct (tungstate, benzyl adenine)			11
11. Cultured tobacco cells	NO_3^-; NO_2^-	direct (kinetics, tungstate)		Casein hydrolysate	29

*Tungstate = induced formation of nitrite reductase by nitrate in the presence of tungstate.

Kinetics = sequential or simultaneous induction of nitrate & nitrite reductase by nitrate.

Benzyl adenine = benzyl adenine has no effect on the induction of nitrite reductase by nitrate.

nitrate reductase (66,74), thus blocking the reduction of nitrate to nitrite.

3. When benzyl adenine, which induces nitrate reductase in some plant systems, was present together with nitrate in culture media, there was no benzyl adenine-dependent increase in nitrite reductase induction.

4. Nitrite reductase was induced by nitrate in nitrate reductase - less mutants of *Aspergillus* (47).

Point 2 needs to be interpreted with caution: Residual nitrate reductase activity, up to 10% of the induced level, was observed in *Azotobacter* (71) and cultured tobacco cells (29), and up to 40% of the induced level in *Agrostemma* embryos (11) when these were exposed in tungstate- and nitrate-containing media.

Indirect induction of nitrite reductase by nitrate was reported in *Anabaena* (44) and radish cotyledons (27) (Table 2). The evidence comes mainly from studies of the kinetics of induction of the enzyme. Sequential induction of nitrate- and nitrite reductases was observed when nitrate was used as inducer, whereas nitrite reductase activity increased immediately in response to the presence of nitrite in the induction media. When nitrate was added

to media containing nitrite and fully induced *Anabaena*, no increase in nitrite reductase activity was observed in the algae (44).

It thus appears that there may be two mechanisms of induction of nitrite reductase. One is represented by *Lemna minor* and other systems where nitrite reductase can be directly induced by either nitrite or nitrate. The other type is observed in *Anabaena* and radish cotyledons where nitrite seems to be the direct inducer. In the second type of induction, the presence of nitrate reductase may be required when nitrate is used to induce the enzyme. This could also explain the sequential appearance of nitrate and nitrite reductases. If this is the case, then nitrate would be a direct inducer but cannot act alone.

The mechanism of induction of nitrite reductase by nitrite and by nitrate might be different. In cultured tobacco cells casein hydrolysate was reported to repress nitrite reductase formation when the enzyme was induced by nitrate, but not when it was induced by nitrite (29).

2. Involvement of nitrate reductase in the induction of nitrite reductase. According to a model proposed by Cove (7), nitrate reductase, when complexed with nitrate, blocks the conversion of an endogenously produced inducer

of nitrate- and nitrite-reductases to a repressor of both of these enzymes (Figure 1). Uncomplexed nitrate reductase, on the other hand, enhances the above conversion. Based on the observation that normal nitrite reductase was induced by nitrate in the presence of tungstate, which prevents the formation of active nitrate reductase, Kelker and Filner suggested that active nitrate reductase was not involved in the induction of nitrite reductase in cultured tobacco cells (29). This interpretation may apply to other systems where similar observations have been made (11,64,71). However, residual nitrate reductase activity has been found in all the above systems except *Lemna minor*.

Cove's model can be used to explain the induction of nitrite reductase in most of the cases reported above. In systems where nitrate and nitrite reductases are induced simultaneously, a low but constant level of nitrate reductase would have to be maintained in the absence of nitrate. When nitrate is present, conversion of the endogenously produced inducer to repressor would be blocked by the nitrate reductase-nitrate complex, causing an increase in nitrate- and nitrite reductase activities. A much higher level of nitrate reductase may be required for such an effect in systems where the sequential appearance of the two enzymes is observed. In nitrate reductaseless mutants, nitrite reductase would be expected

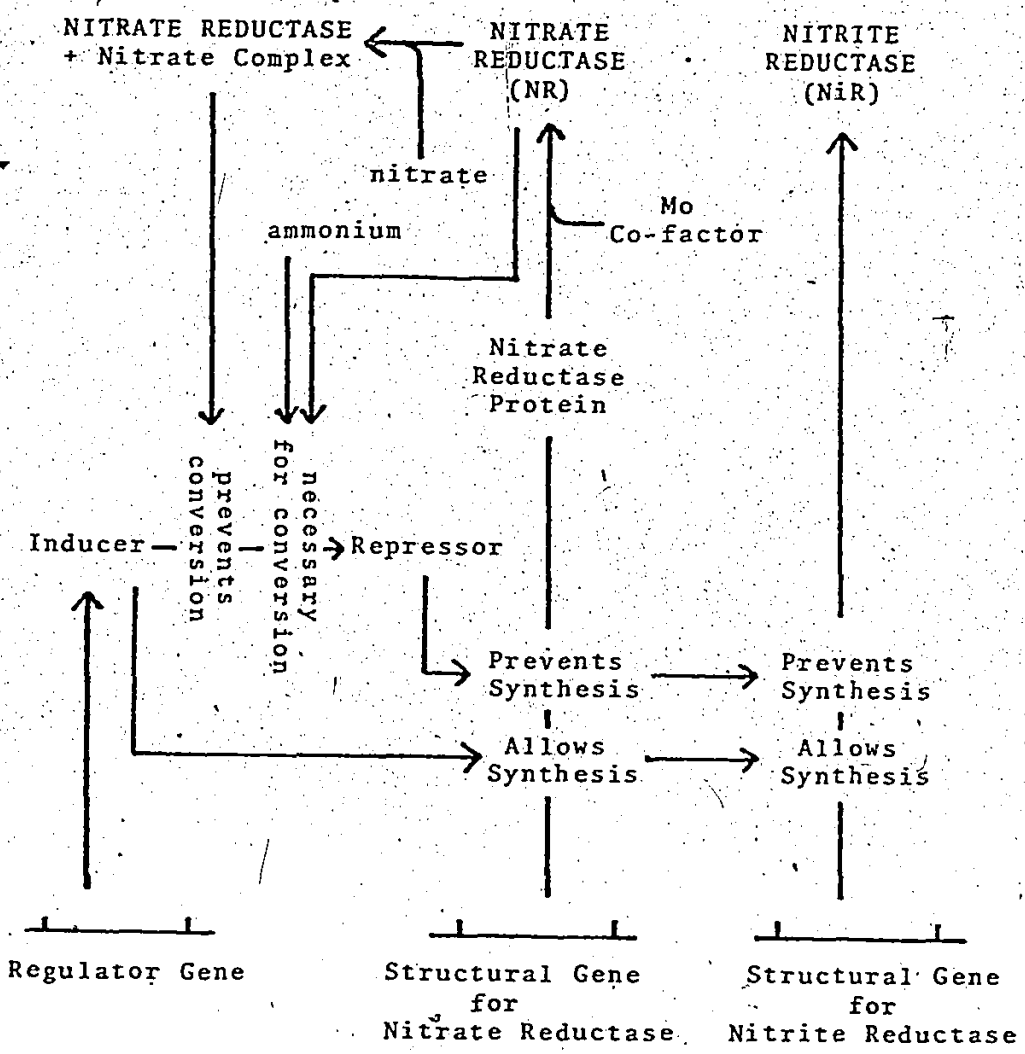


Figure 1. Cove and Pateman's model illustrating the possible mechanisms of the control of the production of nitrate- and nitrite reductase in *Aspergillus nidulans* (7).

to be produced constitutively by Cove's model. This is true of some of the nitrate reductaseless mutants reported in *Aspergillus* (47), but not of others. In the latter cases, inactive nitrate reductase may be produced which behaves like an active nitrate reductase in the conversion of the inducer to repressor as described in Cove's model of regulation.

3. De-repression of nitrite reductase. De-repression of nitrite reductase has been claimed in only one system. Losada *et al.* (34) showed that when *Chlorella* grown on nitrate was transferred to a medium containing ammonia, there was a gradual decrease in the specific activity of nitrite reductase to about 10% of the "induced" level, within 12 hours. The activity increased when the cells were transferred back to a medium containing nitrate. The presence of nitrate in the "de-repression" medium would suggest, that the enzyme may have been induced rather than "derepressed". De-repression should be demonstrated in nitrogen-free medium. Losada *et al.* (34) reported that the "de-repression" required the synthesis of new proteins because it could be inhibited by cycloheximide.

4. Repression of nitrite reductase by ammonia. Nitrite reductase is believed to be susceptible to repression by ammonia. The presence of ammonium ions in nitrate-containing induction media resulted in a lowering of the

final induced level of nitrite reductase activity in a number of systems (13,16,30,34,48,64,71). The extent of repression by ammonium ions varied from none in radish cotyledons (27) to minimal in *E. coli* (30) and *Ditylum* (13), to very substantial in *Aspergillus* (48).

The role of ammonium ions in the repression of nitrite reductase is still unclear. Ammonium ions have been claimed to inhibit the assimilation of nitrate indirectly in *Chlorella*, the reason being that ammonium ions were found not to inhibit the assimilation of nitrate when the algae were starved for a carbon source. The assimilation of nitrate was only partially inhibited by the presence of ammonium ions under comparable conditions in this system (68). Losada *et al.* (34) found that nitrite reductase in *Chlorella*, unlike nitrate reductase, was not inactivated *in vivo* by ammonium ions, suggesting that the inhibition of nitrite reductase by ammonium ions may be indirect. This conclusion is supported by the observation that the presence of 3 mM ammonium chloride in the assay mixture had no effect on nitrite reduction catalyzed by desalted extracts of fully induced *Neurospora* mycelia (40).

The uptake of nitrate or nitrite in the presence of ammonium ions has been studied extensively in *Neurospora* (55,56). Ammonium ions did not induce the uptake systems for nitrate or nitrite, and had a negligible effect on

the induction of these systems by their respective effectors. The rate of uptake of nitrite *in vivo* was not affected by the presence of ammonium ions in the culture medium, while that of nitrate was inhibited by 40%.

5. Repression of nitrite reductase by amino acids and other nitrogenous compounds. Stewart reported that the presence of 13 amino acids in the culture medium could cause between 16 and 87% repression of the induction of nitrite reductase by nitrite, and between 50 and 93% repression of the induction of nitrite reductase by nitrate in *Lemna minor* (64).

Other reported repressors of the induction of nitrite reductase include urea in the case of *Lemna minor* (64); casein hydrolysate in the case of cultured tobacco cells when nitrate was used as inducer (29); and casamino acids in the case of *Neurospora* (56).

6. Mechanism of regulation of nitrite reductase by nitrate and ammonia. Inhibition of nitrite reductase induction by inhibitors of RNA or protein synthesis, such as actinomycin D, cycloheximide or chloramphenicol has been reported in *Chlorella* (32,34), *Lemna* (64), radish cotyledons (27) and rice leaves (53), suggesting *de novo* synthesis of the enzyme during induction. It is not known if the effect of the "inducer(s)" and of the "repressor(s)" is at the level of transcription, of

translation or at a post-translational step.

In the model proposed by Cove and co-workers (7) for the regulation of nitrate and nitrite reductases in *Aspergillus*, it was suggested that nitrate and ammonium ions exerted antagonistic effects on the conversion of an endogenously produced inducer to a repressor, thus indirectly regulating the synthesis of the two reductases (Figure 1). In this way, nitrate and ammonium ions were functioning at the level of transcription. However, there is no direct evidence supporting this model.

7. Stability of nitrite reductase *in vivo*. The stability of the reductase has been studied in cultured tobacco cells (29). When cells exposed to nitrate are transferred to a medium containing no nitrogen source, nitrite reductase activity decays with an initial half-life of approximately 28 hours, followed by a slower decay, with a half-life of approximately 124 hours. This latter rate was similar to the rate of decay of the enzyme in cells left in nitrate medium after induction.

D. Conclusion

Studies of nitrite reductase have shown that ammonia is a product of the catalytic reduction of nitrite reduction by this enzyme. In some systems, nitrite is not reduced to ammonia stoichiometrically, suggesting the presence of more than one nitrite-reducing activity.

Both direct and indirect induction of nitrite reductase by nitrate have been reported. The role of ammonium ions in the repression of the enzyme is not known.

The experiments reported in this thesis are intended to explore the reasons for the lack of stoichiometry between nitrite reduction and ammonia production catalyzed by crude extracts of *Neurospora*. The role of nitrate as an "inducer" has been studied. The role of ammonium ions in the repression of the enzyme has also been examined.

MATERIALS AND METHODS

A. Fungal Strains

Neurospora crassa strain *74A* was used as the wild type strain. Strain *nit-6* which was isolated and characterized by C. Dyer (12) cannot utilize nitrite, but can utilize ammonia as a source of nitrogen. Strains *nit-1*, *nit-2*, *nit-3*, *nit-4* and *nit-5* have been described previously (3,61). Strains *am-1*, *am-2* and the *alcoy* tester were obtained from the Fungal Genetics Stock Centre, California State University, Humboldt, Arkata, California.

B. Media

The basic medium used was that described by Sorger and Giles (59). In addition, nitrate medium contains potassium nitrate (2 g/l), nitrite medium contains sodium nitrite (0.5 g/l), ammonia medium contains ammonium tartrate (4 g/l), and casamino acids (CA) medium contains casamino acids (5 g/l) as nitrogen sources, unless stated differently. Medium containing no nitrogen source is designated as no nitrogen medium. When sodium nitrite was used as source of nitrogen, it was sterilized separately and the culture medium was neutralized with sodium bicarbonate.

C. Culture Conditions

Erlenmeyer flasks of 250 ml capacity, containing 50 ml of ammonia medium (or CA medium for *am* strains) were inoculated with *Neurospora* conidia. The cultures were left standing in an incubator at 27°C for 72 hours. The resulting mycelial pads, in their late log phase, were then harvested, washed with distilled water, transferred to designated media and incubated in a rotary shaker for 8 - 10 hours at 27°C.

D. Extraction

The "induced" mycelial pads were harvested, washed with distilled water, and blot-dried with paper towels. The pads were then frozen in liquid nitrogen and subsequently ground with approximately an equal weight of silica powder and 3 times their weight of ice cold 0.1 M potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA (EDTA was absent from the buffer used to extract nitrate reductase). The slurry was centrifuged at 16,000 X g for 20 minutes in a refrigerated centrifuge (Sorvall RC-2B). The resulting supernatant was kept on ice until used as the crude extract for assays of enzyme activity. The frozen pads can be stored at below -40°C for 2 weeks without losing activity.

E. Assay of Nitrite Reductase Activity

Nitrite reductase was assayed as described by Cook (6) with some modifications. The reaction mixture contained in a final volume of 2.9 ml: potassium phosphate pH 7.5, 120 µmoles; methyl viologen, 0.5 µmoles; sodium nitrite, 4.5 µmoles; and 0.05 to 0.4 ml of extract. The reaction was initiated by adding 0.5 ml of extraction buffer containing 7.5 µmoles each of sodium hydrosulfite and sodium bicarbonate, the tubes were then gently shaken and incubated in a water bath at 30°C for 10 to 20 minutes. One tenth of a millilitre of 25% barium acetate was added at the end of the reaction, the assay mixture was then oxidized by agitation on a Vortex mixer until colorless, and subsequently stored in the freezer for 10 minutes before spinning in a bench top centrifuge. The clear supernatant was used for the determination of nitrite and ammonia. An assay mixture incubated as the test, but containing no crude extract (or filtrate, or acetone-supernatant) or containing no nitrite, was used as a blank in the determination of the activity which reduces nitrite (ND), or of the activity which produces ammonia (AP), respectively. One unit of ND activity, or AP activity is defined as the disappearance of one nanomole of nitrite, or the appearance of one nanomole of ammonia per minute, respectively.

Measurement of the concentration of nitrite: The method was adapted from Sanderson and Cocking (51). One ml each of 1% (w/v) sulphanilamide in 1N HCl and 0.01% (w/v) n-1-naphthylethylene diamine dihydrochloride in distilled water were added to 0.1 ml of assay mixture plus 7.9 ml distilled water. The resulting magenta color was estimated after twenty minutes in a Klett-Summerson Colorimeter equipped with a #54 filter. The concentration of nitrite was determined by relating the intensity of the color to a standard curve (Figure 2). There is a linear relationship between Klett units under such conditions and nitrite concentration from 0 to 200 nmoles per reaction mixture.

Measurement of the concentration of ammonia: A modification of the Conway microdiffusion method described by Mulkins was used (40). A two ml aliquot of the oxidized clear assay mixture was placed in the outer well of a Conway microdiffusion disc and 1.5 ml of saturated potassium carbonate solution was then added. Ammonia was allowed to diffuse for four hours into the centre well which contained 1.5 ml of 2% boric acid. The content of ammonia in a one ml aliquot of the boric acid was then measured by adding 2 ml of Nessler's reagent and reading the resultant color at 440 nm in a Beckman DBG spectrophotometer after 20 minutes. The measurements were related to a standard curve (Figure 3).

The error in the measurement of nitrite reduction or of ammonia production is 1.5 and 2.7 units, respectively.

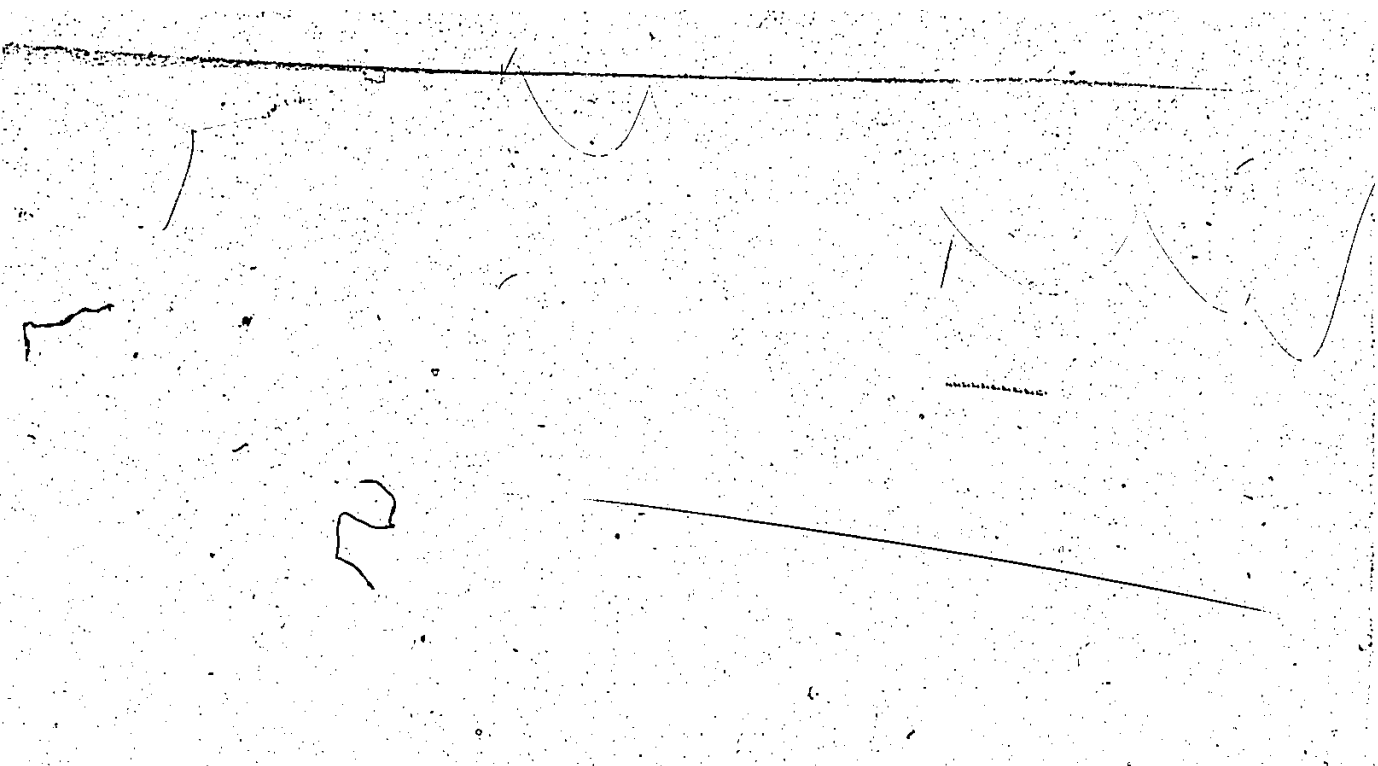


Figure 2. Standard curve for nitrite determination.

Sodium nitrite solutions of different concentrations were made with double-distilled water. Aliquots of 0.1 ml were used to react with sulfanilamide and N-1-napthylethylenediamine dihydrochloride as described in Methods. The resulting color was read in a Klett-Summerson Colorimeter with a number 54 filter. For determination of nitrite content of the assay mixture, the value from the curve was multiplied by 3.

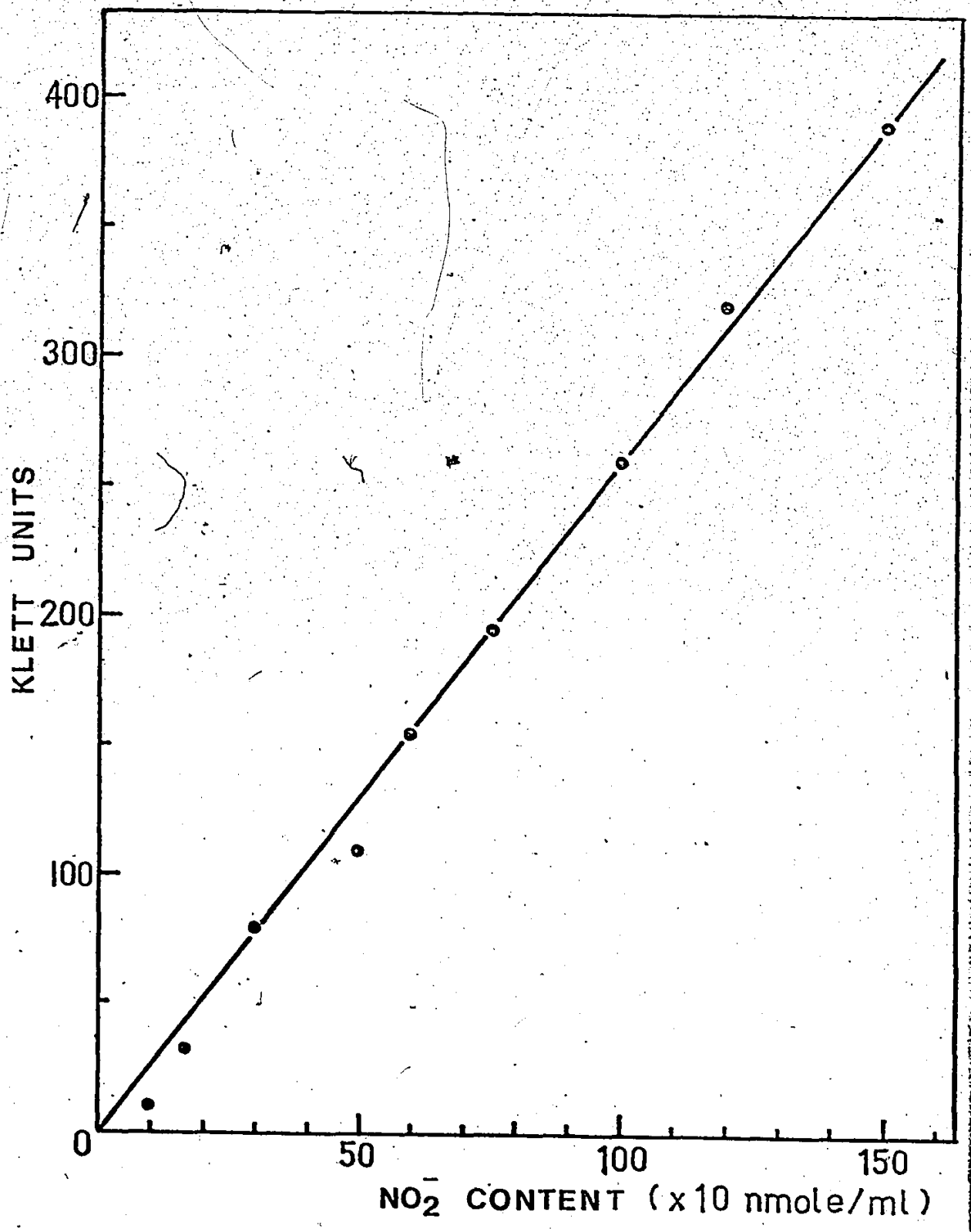
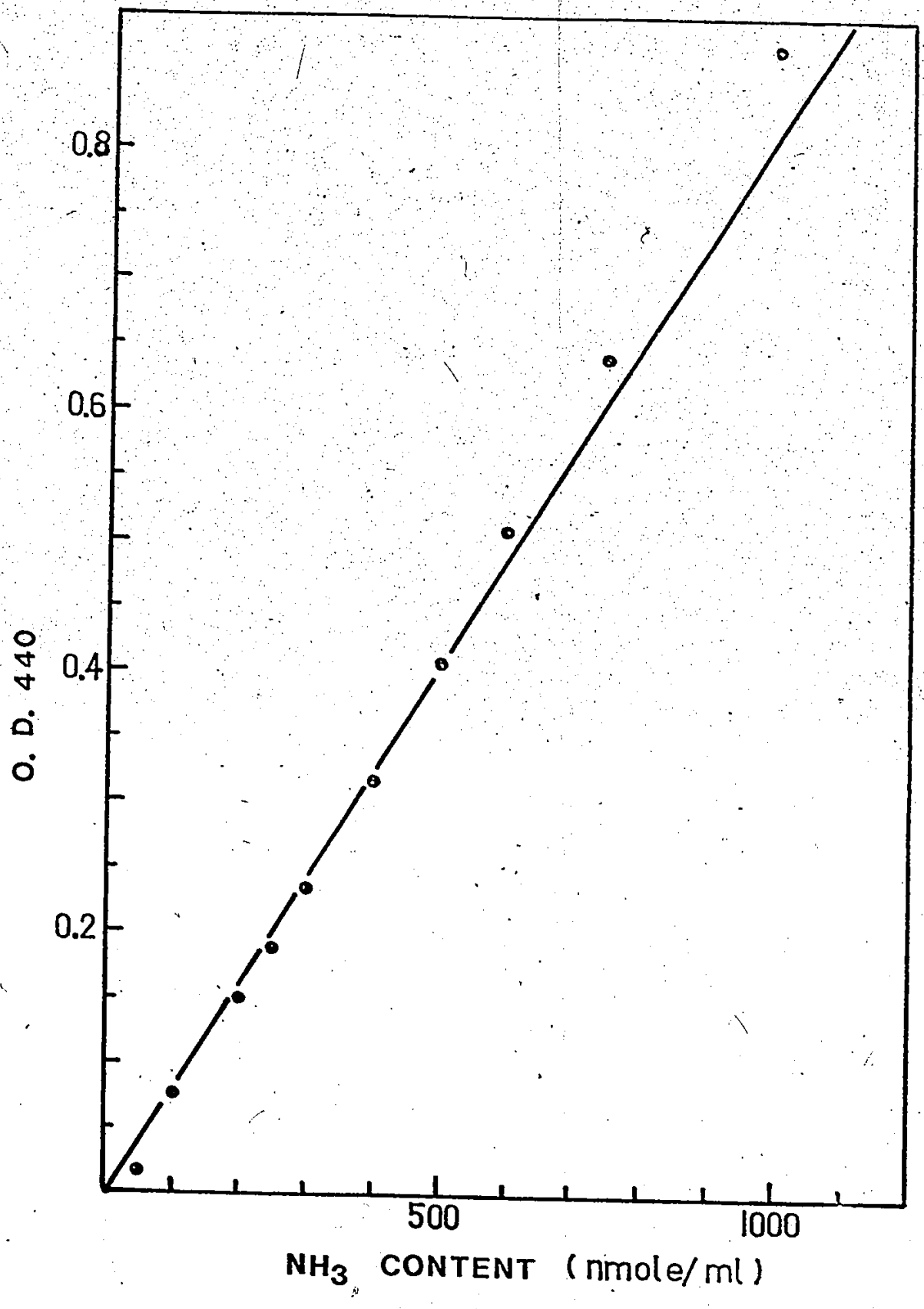

400
300
200
100
0
KLETT UNITS
50
100
150
NO_2^- CONTENT (x 10 nmole/ml)

Figure 3. Standard curve for ammonia determination.

Ammonium chloride solutions of different concentrations were made with double-distilled water. Aliquots of one ml were used in the Conway diffusion disc and subsequently reacted with Nessler's reagents as described in the Methods. The optical density at 440 nm was determined on a spectrophotometer. For determination of ammonia content of the assay mixture, the value from the curve was multiplied by $\frac{9}{4}$.


0.8
0.6
0.4
0.2
O. D. 440
500
1000
NH3 CONTENT (nmole/ml)

F. Assay of Nitrate Reductase Activity

NADPH-nitrate reductase was assayed as described by Sorger (60) with some modifications. The assay mixture contained in final volume of 0.5 ml: sodium pyrophosphate buffer (pH 7.0), 57 µmoles; potassium nitrate, 11.4 µmoles; FAD, 0.6 nmoles; NADPH, 0.1 µmoles; and 0-0.1 ml of extract. NADPH was added last to initiate the reaction which was incubated at 30°C. The reaction was stopped by adding 0.1 ml of 25% (w/v) barium acetate which precipitates the pyridine nucleotides and subsequently the mixture was spun in a bench top centrifuge for 10 minutes. The use of barium acetate to precipitate pyridine nucleotides in this assay was found to be adequate. The effect of this procedure is no different from the use of an excess of glutamate dehydrogenase plus α-ketoglutarate to remove the interference of reduced pyridine nucleotide in nitrite determination (unpublished result of G.J. Sorger). The content of nitrite of the supernatant was determined by adding 4 ml of color reagent mixture composed of 1 part of 1% (w/v) sulphanilamide in 1N HCl, 1 part of 0.01% (w/v) n-1-napthylethylene diamine dihydrochloride and 3 parts of distilled water; the resultant color was read at 540 nm in a Beckman DBG spectrophotometer after 20 minutes. The measurement was related to a standard curve made with sodium nitrite. One unit of nitrate reductase

activity is defined as the appearance of one nanomole of nitrite per minute.

G. Assay of Glutamate Dehydrogenase Activity

Glutamate dehydrogenase was assayed as described by Sanwal and Lata (52). The reaction mixture contained in a final volume of 3 ml: 20 µmoles α-ketoglutarate; 120 µmoles ammonium sulphate; 273 µmoles Tris buffer pH 7.5; and 0-0.2 ml of extract. The reaction was initiated by adding 0.1 ml of NADPH (2.8 mg/ml) in 0.1 M Tris buffer pH 7.5, and carried out in a cuvette at 30°C. The oxidation of NADPH was measured continuously at 340 nm in a 1 cm light path in a Beckman DBG spectrophotometer coupled to a recorder. A reaction mixture containing no α-ketoglutarate was used in the reference cuvette as a blank. One unit of the enzyme activity is defined as the oxidation of one nanomole of NADPH per minute.

H. Measurement of the Concentration of Protein

The concentration of protein was determined by the Biuret method (10) with bovine serum albumin as standard. When fractions were being eluted from a Sephadex column the protein concentration of the fractions was monitored by measuring the optical density of samples at 280 nm.

I. Determination of the Content of Nitrate in Extracts of Mycelia

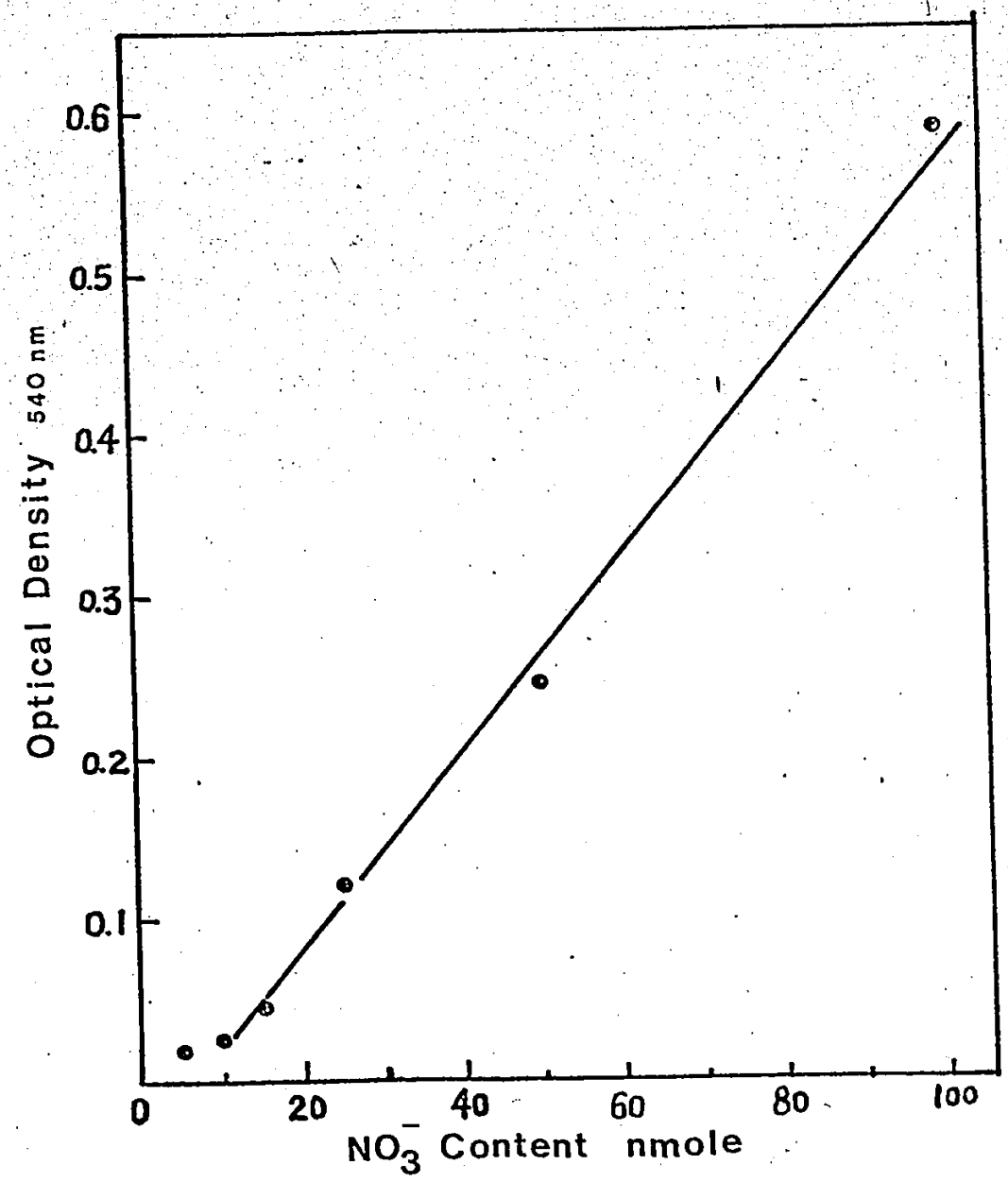
The content of nitrate of extracts was measured by reducing the nitrate to nitrite with NADPH-nitrate reductase, and determining the resultant nitrite colorimetrically (67). The assay mixture contained 0.4 ml boiled extract which had been clarified by centrifugation, 0.4 ml of 0.2 M sodium pyrophosphate buffer pH 7.0 containing 1.5 µM FAD, and 0.1 ml active nitrate reductase preparation. The reaction was initiated by adding 0.1 µmole NADPH, and the mixture was subsequently incubated at 30°C for 20 minutes. The reaction was stopped by the addition of 0.1 of 25% (w/v) barium acetate, and the assay mixture subsequently centrifuged. The content of nitrate of the supernatant was determined colorimetrically and related to a standard curve made with potassium nitrate (Figure 13).

J. Determination of the Content of Ammonia in Extracts of Mycelia

The content of ammonia in one ml of extract was determined with the Conway microdiffusion method and Nessler's reagent as described before.

Figure 13: Standard curve for nitrate determination.

Different amounts of potassium nitrate were used in nitrate reductase assay. The assay mixture was composed of: 0.05 ml active nitrate reductase preparation, 0.2 ml of 0.2 M sodium pyrophosphate buffer pH 7.0 containing 1.5 µM FAD, 0.15 ml of potassium nitrate solution of different concentrations. The reaction was initiated by adding 0.1 µmole NADPH, and the mixture incubated at 30°C for 20 minutes. The reaction was stopped by the addition of 0.1 ml of 25% (w/v) barium acetate, and the assay mixture subsequently centrifuged. The supernatant was reacted with sulfanilamide and n-1-napthylethylene diamine dihydrochloride as described in Methods. The resultant color was read in a Beckman DBG spectrophotometer after 20 minutes.


Optical Density 540 nm
0.6
0.5
0.4
0.3
0.2
0.1
0
20
40
60
80
100
NO3− Content nmole

K. Column Chromatography

Sephadex gel was used. The gel was first swollen in distilled water at room temperature for 2 days, and subsequently equilibrated with the extraction buffer by washing the gel with buffer five times. After each wash, the gel suspension was allowed to settle for a few minutes and the supernatant which still contained fine gel particles was decanted. The buffered gel suspension was then deaerated with a vacuum pump and subsequently cooled to 4°C for packing. The gel was poured into the column in one lot with the use of a reservoir attached to the top of the column and allowed to settle for 5 hours before eluting by gravity at a flow rate of 10 ml/hour. Two sizes of column were used: 2.5 cm x 35 cm and 2.2 cm x 25 cm.

The sample (less than 2% of the total column volume) was applied onto the column through a feeding tube to avoid disturbance of the gel surface. Elution was with 0.1 M potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and the flow rate was regulated at 15-20 ml/hour. Fractions were collected by a Buchner Fractometer II.

L. Ultrafiltration

Crude extracts were introduced into a collodion bag, and filtered with the aid of a vacuum pump. The filtrate and concentrated extract were collected, and kept

on ice until assayed for nitrite reductase activity.

M. Acetone Precipitation

Crude extracts were mixed with an equal volume of acetone, precooled to -20°C in an ice bucket and stirred for 10 minutes. The mixture was allowed to stand for an additional 5 minutes and subsequently centrifuged at 6,000 X g for 10 minutes at 0-4°C in a refrigerated centrifuge. The supernatant and precipitate were lyophilized overnight, subsequently resuspended in ice-cold distilled water, and kept at 0-4°C until assayed for nitrite reductase activity.

N. Materials

Benzyl viologen was from Mann Research Labs., New York; methyl viologen, NADPH, FAD, bovine serum albumin, 2,2' bipyridine, o-phenanthroline, p-chloromercurobenzoate, α-ketoglutarate, proteases Form VI and VIII were obtained from Sigma Chemical Co., St. Louis, Mo.; pepsin was from Pentex Biochemicals, Kankakee, Ill.; Trypsin was from Mile-Seravac (PTY) Ltd., Maidenhead, Berks, England; Nessler's reagent was from Paragon Co., Bronx, N.Y.; Sulphanilamide and N-1-Naphthylethylene diamine dihydrochloride were products of Eastman Organic Chemicals, Rochester, N.Y.

Collodion ultrafiltration membranes were purchased from Glaxo (Canada) Ltd., Toronto, Ontario.

All inorganic reagents were of analytical grade and were obtained from Fisher Scientific Co., Fairlawn, N.J., or from Baker Chemical Co., Phillipsburg, N.J.

RESULTS

A. The Nitrite Reductase Activity That Does Not Catalyze The Production of Ammonia

1. Lack of stoichiometry and multiplicity of activities.

When crude extracts of wild type mycelia, which had been exposed to nitrate (or nitrite) for 8 hours, were used to catalyze the reduction of nitrite, between 60 and 85% of the nitrite reduced was recovered as ammonia (Table 3). This result confirms the lack of stoichiometry between nitrite reduction and ammonia production reported by Mulkins (40).

Crude extracts of wild type mycelia, which had been exposed to ammonia-medium or to no-nitrogen medium, did not catalyze the reduction of nitrite to ammonia (Table 3), but did contain a nitrite-reducing activity. The observation supports the suggestion that there are more than one nitrite reducting activity present in extracts of *Neurospora* mycelia (40). Additional support comes from studies with strain *nit-6*, a mutant which cannot grow on nitrite or nitrate as sources of nitrogen, but which can assimilate ammonium ions. Extracts of *nit-6* mycelia, which had been exposed to any of the nitrogen sources tested, contain nitrite reducing activity but no

Table 3

Nitrite Reducing and Ammonia Producing Activities Present in Extracts of Wild Type and *nit-6* Mycelia Exposed to Different Nitrogen Sources

Strain	N-source in induction medium	Specific activity (units*/mg protein) NO_2^- reduction (a)	NH_4^+ production (b)	b/a
Wild type	NO_2^- (10 mM)	14.1	8.8	0.62
	NO_3^- (10 mM)	24.8	19.3	0.78
	NH_4^+ (10 mM)	10.9	<0.5	<0.05
	-N	15.6	0	0
nit-6A	NO_3^- (10 mM)	11.2	0	0
	NH_4^+ (10 mM)	7.2	0	0
	-N	12.3	0	0

* nmole per min.

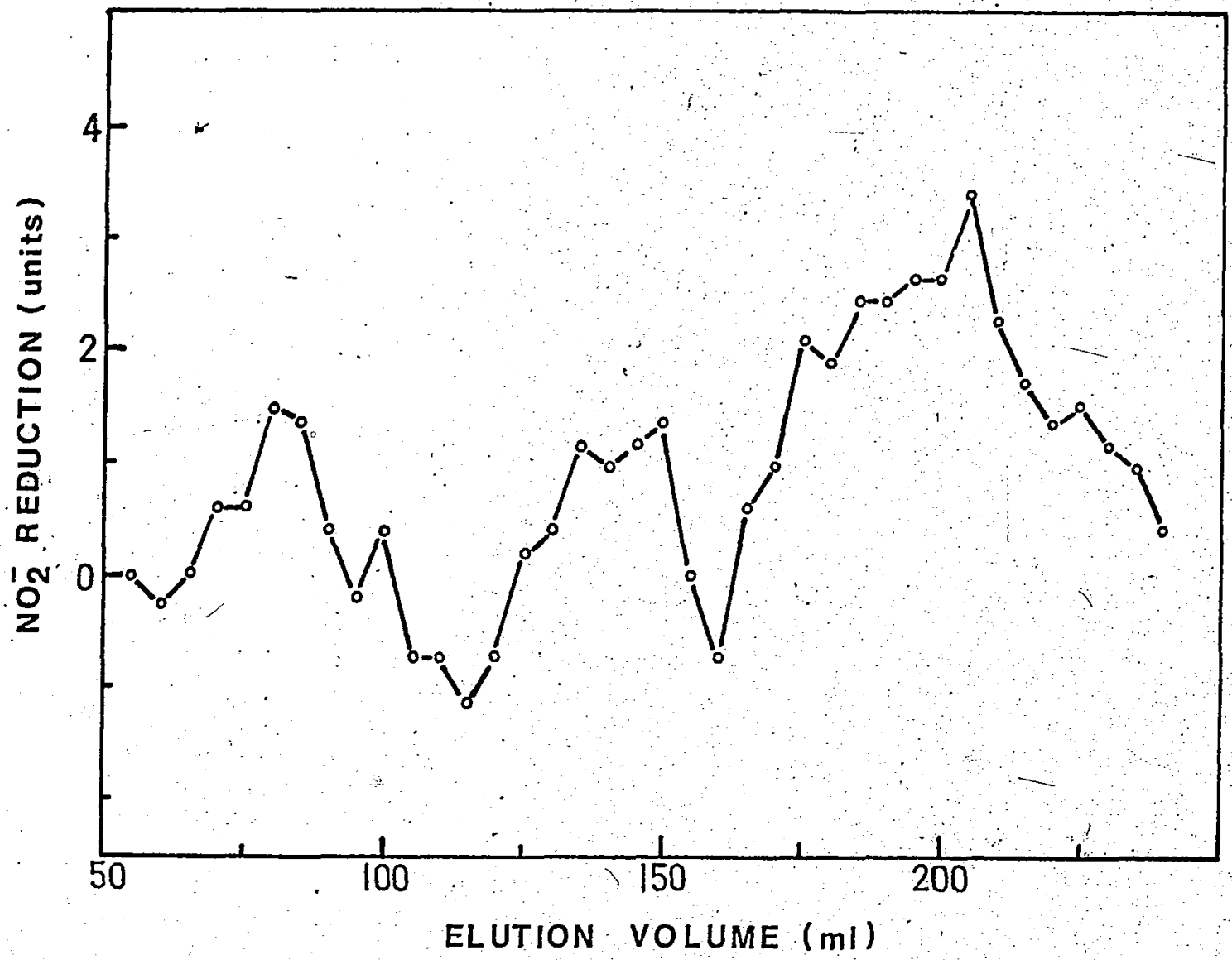
Extracts were prepared from mycelia exposed to different nitrogen sources as described in Methods. The results shown are representative of six experiments.

ammonia producing activity. (Table 3). These results suggest that there may be two activities which reduce nitrite: one, present in the wild type strain and absent in *nit-6*, which is inducible by nitrate and by nitrite, and which catalyzes the production of ammonia from nitrite (AP activity); and another which is present in all mycelial extracts, is not inducible, and which reduces nitrite to something other than ammonia (ND activity).

2. Isolation of the low molecular weight ND activity. If the two nitrite reducing activities reside on molecules of different sizes, they should be separable by Sephadex gel chromatography. When extracts of wild type mycelia which had been induced on nitrate medium were applied to a Sephadex G-100 column, the elution pattern appeared to reveal three peaks of nitrite reducing activity, but the peaks were not reliably higher than the background variation (Figure 4). Crude extracts were then concentrated before being put onto the column. Several concentration procedures were used: ultrafiltration of the extracts with an Amicon membrane; precipitation of the extracts with ammonium sulphate; ultrafiltration of the extracts through a column of Sephadex G-25 beads to retard the small molecules. None of these methods of concentration were satisfactory, because there was too much loss of activity. Since most of the concentration procedures

Figure 4. Behaviour of nitrite reductase during gel exclusion chromatography through a column of Sephadex G-100.

Four ml of crude extract of fully induced wild type mycelia was put onto a column (2.5 cm x 35 cm) containing 172 ml of Sephadex G-100 gel. The eluant was collected in 5 ml fractions and 0.5 ml aliquots were subsequently assayed for nitrite reducing activity. The void volume was 75 ml.


4
2
0
NO_2^- REDUCTION (units)
50
100
150
200
ELUTION VOLUME (ml)

removed small molecules, the loss of activity was postulated to be due to the loss of some small molecule(s) containing, or necessary for nitrite reducing activity.

Ultrafiltration was first used to isolate the small molecules that had ND activity from crude extracts of fully induced mycelia. Later, ultrafiltration was replaced by precipitation of the crude extracts with 50% acetone. The effect of the two isolation procedures is shown in Tables 4 and 5, respectively. Both the ultrafiltrate and the supernatant of the acetone precipitation contained ND activity, but no AP activity.

3. Recovery of ND and AP activities from crude extracts using different isolation procedures. Eighty percent of the original ND activity contained in the supernatant of an acetone-treated crude extract was filterable through a collodion bag (Table 5). The recovery of the nitrite reducing activity from ultrafiltration was estimated in a way which would simulate what happens when a crude extract is subjected to ultrafiltration. Two preparations consisting of a) a mixture of equal volumes of crude extract and of extraction buffer, and b) a mixture of equal volumes of crude extract and of a supernatant from an acetone-treated crude extract were passed separately through a filtration membrane. The difference in the content of ND activity between the two filtrates is due to the quantity of filterable activity contributed by

Table 4

The Effect of Ultrafiltration on the Nitrite Reducing and Ammonia Producing Activities Present in Crude Extracts of Wild Type Mycelia Exposed to Nitrate

Preparation	Activity (units/0.4 ml) nitrite reduction	ammonia production
Crude extract	29.4	18.0
	30.3	not done
Crude extract concentrated 2-fold by ultrafiltration	17.5	18.8
	15.5	not done
Filtrate	10.8	0
	11.5	0

Mycelia were grown, induced and extracted as described in Methods. Ten ml of crude extract were filtered through a collodion bag. The nitrite reducing and ammonia producing activities of 0.4 ml of the untreated extract , of the extract concentrated to 5 ml in the collodion bag, and of the filtrate were assayed. The figures shown are representative of three experiments.
The activity of the filtrate has been multiplied by 1.25 to account for the 20% loss of filterable activity (see Table 6) due to filtration.

Table 5

Filterability of the Nitrite Reducing and Ammonia Producing Activities Residing in the Supernatant and in the Precipitate of Fully Induced Wild Type Extract Which Has Been Treated With Acetone

Fraction	Activity (units/0.4 ml) Unfiltered nitrite reduction	Unfiltered ammonia production	Filtrate nitrite reduction
Crude extract (10 ml)	34.5	16.4	not done
Precipitate freeze dried and resuspended in 5 ml H_2O	18.0	8.6	0 (0)
Supernatant freeze dried and resuspended in 10 ml H_2O	13.2	<0.5	10.6 (80)

10 ml crude extract was incubated in an ice bath for 20 minutes with an equal volume of acetone precooled to -20°C. The mixture was subsequently centrifuged at 6000 g for 10 minutes and the supernatant and the precipitate were freeze dried separately and subsequently resuspended in 10 ml and in 5 ml of ice cold distilled water, respectively. Aliquots of the resuspended preparations were filtered through a collodion membrane. Nitrite reducing and ammonia producing activities were then measured. Numbers in parentheses represent the recovery of activity as a percentage of the original unfiltered activity. The results shown are representative of four experiments.

the supernatant of the acetone-treated extract. Eighty percent of the ND activity present in the mixture of crude extract plus the supernatant of the acetone-treated extract was filterable (Table 6). This recovery is similar to that obtained when the supernatant of the acetone-treated extract was filtered alone. If one assumes that all of the ND activity present in the supernatant of the acetone-treated extracts is potentially filterable, then it would appear that 20% of this activity is not recovered after filtration regardless of the presence or absence of other extract components during filtration.

The recovery of ND activity from crude extracts treated with acetone was 70% of the calculated original activity in the crude extracts (Table 7). This figure was obtained as follows: a crude extract of fully induced wild type mycelia was divided into two portions; one was treated with acetone, the other, serving as a reference, was filtered through a collodion membrane. The concentration of nitrite-reducing activity in the two preparations was then measured and compared, using the figure of 80% recovery for the ultrafiltered preparation as the standard.

The difference between the nitrite reduced (30.3 units) and the ammonia produced (18.0 units) catalytically in the presence of crude extracts of fully induced wild type mycelia could be accounted for as ND activity (11.5 units) (Table 4). A similar difference shown in Table 5 can also be accounted for by the ND activity. It would

Table 6

Recovery of Nitrite Reducing Activity Isolated from Crude Extracts of Fully Induced Wild Type Mycelia by Ultrafiltration

Expt.	Nitrite reduction (units/0.4 ml)				% Recovery
	Fa	Fb	S	Fb-Fa	*R*
1	9.0	21.3	15.9	12.3	79
2	7.2	15.6	10.2	8.4	82

Four ml of a crude extract of fully induced wild type mycelia were mixed with a) 4 ml of extraction buffer, b) 4 ml of supernatant from crude extract of fully induced mycelia treated with acetone as described in Table 5. The two mixtures were filtered separately through a collodion bag. The nitrite reducing activity of the two filtrates (Fa and Fb, respectively), and of the supernatant of the acetone-treated preparations (S) above were determined. The % recovery (*R*) of nitrite reducing activity was calculated according to

$$R = \frac{(Fb-Fa)}{S} \times 100$$

The results shown are representative of three experiments.

Table 7

Recovery of Nitrite Reducing Activity from a Crude Extract of Fully Induced Wild Type Mycelia Treated with Acetone

Expt.	Nitrite reduction (units/0.4 ml)		%
	Filtrate (F)	Acetone treated preparation (S)	recovery (*R*)
1	11.3	7.6	67
2	9.1	6.5	72

Aliquots of crude extract were a) filtered through a collodion bag, and b) treated with cold acetone as described in Table 5. The content of nitrite reducing activity of the filtrate (F) and of the supernatant from acetone precipitation (S) were measured. The % recovery of nitrite reducing activity was calculated according to $R = \left(\frac{S}{F}\right) 100$. The activity of the filtrate has been multiplied by 1.25 to account for the loss of activity due to filtration. The results shown are representative of three experiments.

appear, consequently, that the nitrite reductase which is not filterable through a collodion membrane but which is precipitable by acetone, catalyses the stoichiometric reduction of nitrite to ammonia. When a crude extract was concentrated by ultrafiltration, it reduced nitrite stoichiometrically to ammonia, but the recovery after ultrafiltration was only 50% of the original activity (Table 4). The precipitate resulting from the treatment of crude extracts of fully induced wild type mycelia with acetone contains far less nitrite reducing activity than the crude extract from which it was prepared, and contains little AP activity. The nitrite reducing activity present in this precipitate was not filterable (Table 5). The acetone treatment may have extensively inactivated both the filterable and nonfilterable activities.

4. Properties of the ND activity. The molecular weight of the ND activity in the supernatant of extracts treated with acetone is estimated to be approximately 600 due to its gel exclusion chromatographic behaviour (Figure 5).

The ND activity was found not to be precipitable by 10% trichloroacetic acid or by 95% ethanol.

The ND activity is not extractable into non-aqueous solvents (Table 8). When the aqueous supernatant from crude extracts treated with acetone was mixed and shaken with chloroform, or with petroleum ether, the ND activity was recovered from the aqueous phase with no loss. The lower

Figure 5. Behaviour of ND activity during gel exclusion chromatography through a column of Sephadex G-25.

The inset shows the relationship between K_{AV} value and molecular weight of the markers - ATP, Vitamin B_{12}, Insulin and the estimated size of the ND activity.

Two ml of acetone-extracted preparation of fully induced wild type mycelia was put onto a column (2.2 cm X 23 cm) containing 81 ml of Sephadex G-25 beads. The eluant was collected in 4 ml fractions and subsequently 0.5 ml aliquots were assayed for nitrite reducing activity. A 2 ml mixture containing 1 mg Vitamin B_{12} (V_{B12}), 2 mg insulin (Ins.) and 10 mg ATP was used to standardize the column. The volume volume (V_o) was 34 ml.

Symbols: Δ ND activity.

o optical density at 280 nm.

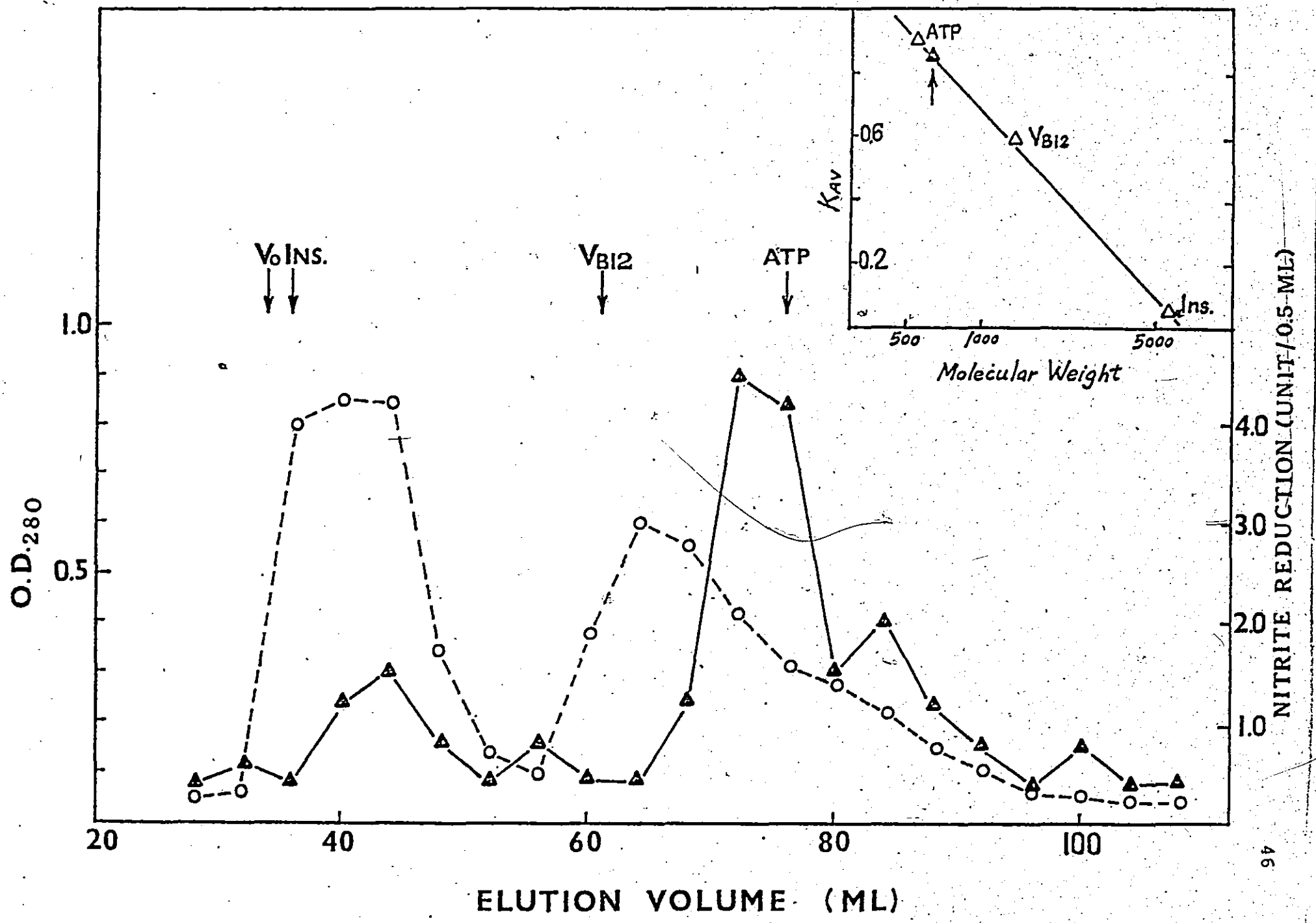

Vo INS.
VB12
ATP
O.D.280
NITRITE REDUCTION (UNIT/0.5 ML)
ELUTION VOLUME (ML)
1.0
0.5
20
40
60
80
100
4.0
3.0
2.0
1.0
ATP
VB12
Ins.
0.6
0.2
KAV
500
1000
5000
Molecular Weight

Table 8

Effect of Organic Solvents on Nitrite Reducing Activity in Fully Induced Wild Type Mycelial Extract Treated with Acetone

Treatment	Nitrite reducing activity (units/0.5 ml of aqueous phase)
Untreated	23.7
Chloroform	23.7
Petroleum ether	26.0
Methanol*	15.0

A 2 ml aliquot of extract treated with acetone was mixed with the same volume of each of the solvents shown above, and shaken for 3 minutes. The mixture was allowed to settle into two phases (where applicable). One half of a ml of the aqueous phase was assayed for nitrite reducing activity. No activity was found in the non-aqueous phase. The results shown are representative of three experiments.

* No aqueous layer

activity observed with the methanol extraction was due to dilution by methanol of the aqueous supernatant.

To determine whether the ND activity resided in a polypeptide, the supernatant of crude extract of fully induced mycelia treated with acetone was preincubated with proteases before being assayed. Preincubation with pepsin, and Sigma protease Form VIII for 3 hours at 24C, caused a reduction of 50% of the ND activity (Table 9). Trypsin and Sigma protease Form VI had no effect under comparable conditions. The effect of pepsin should be interpreted with caution due to the fact that the optimal pH for pepsin activity is 3 (54), whereas the incubation with the supernatant was at pH 7. The loss of activity does not seem to be due to the non-specific binding of the ND activity to proteins, since incubation of the supernatant of acetone-extracted fully induced wild type mycelia with bovine serum albumin resulted in no loss of activity. This was further checked by preincubating the supernatant of acetone extracted fully induced mycelia with Sigma protease Form VIII for 12 hours, and subsequently subjecting it to gel exclusion chromatography on a column of Sephadex G-25. The ND activity did not elute with the protease (Figure 6). Instead there was a slight shift of the position of the peak of ND activity in the elution profile, suggesting that the ND activity now resided on a smaller molecule. This shift could be the result of a change in the adsorption to Sephadex, however, so no firm

Table 9

Effect of Proteases on Nitrite Reducing Activity in Fully Induced Wild Type Mycelia Extracted with Acetone

Treatment	Nitrite reducing activity (units/0.5 ml)
Untreated	20.2
Trypsin	18.0
Pepsin	10.1
Sigma Protease VI	20.2
Sigma Protease VIII	10.1

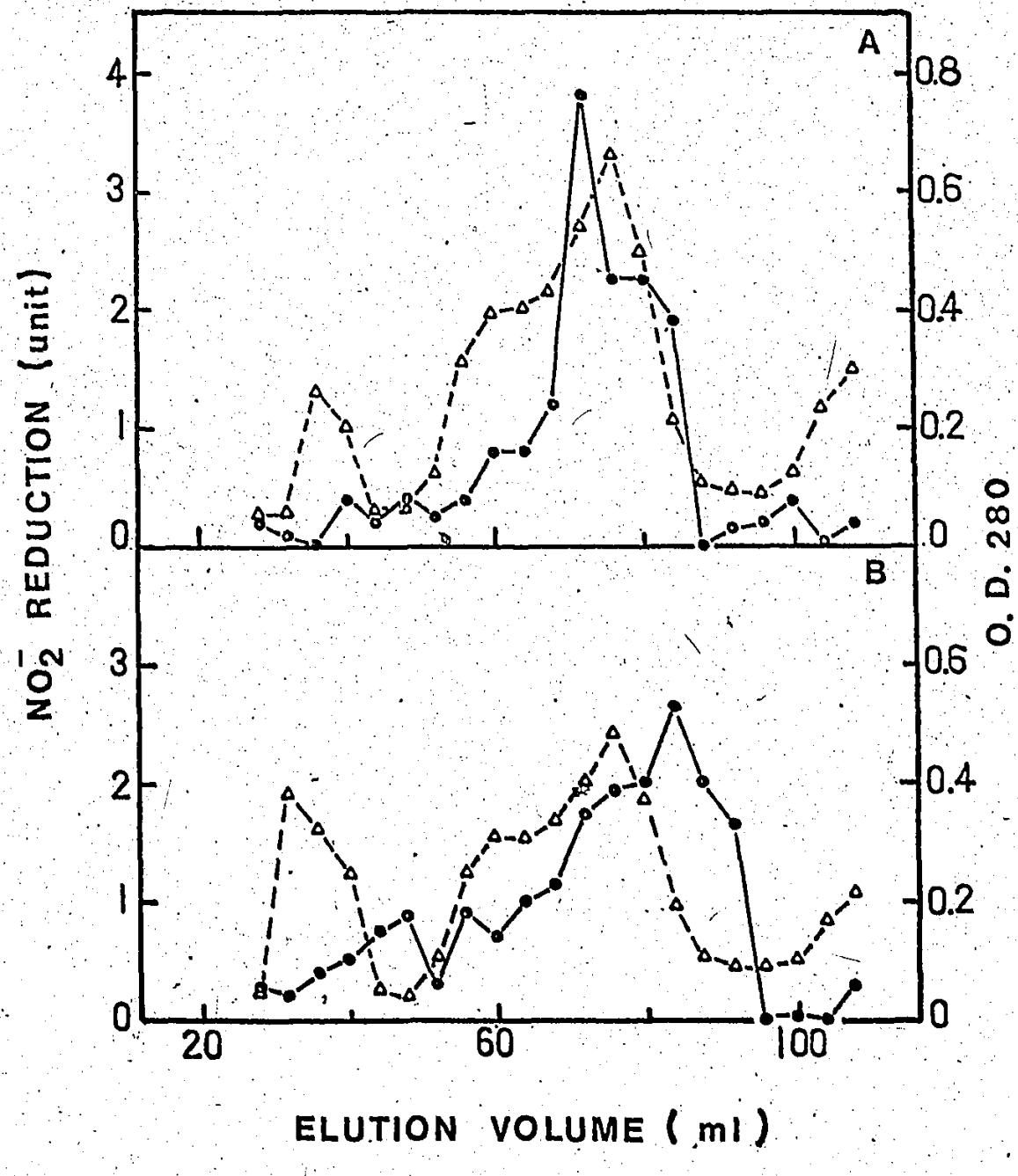
Aliquots of acetone-extracted preparations of fully induced wild type mycelia were pre-incubated with 1 mg/ml of the proteases indicated above for 3 hours at 24°C at pH 7.0. The results shown are representative of three experiments.

Figure 6. Behaviour of ND activity in an acetone-extracted preparation of fully induced wild type mycelia during gel exclusion chromatography before (A) and after treatment with 1 mg/ml of Sigma Protease Form VIII for 12 hours at 24°C (B).

Two ml of the treated or untreated preparation from an acetone treated extract of fully induced wild type mycelia was put onto a column (2.2 cm X 23 cm) containing 86 ml of Sephadex G-25 gel. The eluant was collected in fractions of 4 ml and subsequently assayed for nitrite reducing activity. The supernatant used in (B) had been pre-incubated with Sigma protease form VIII (1 mg/ml) for 12 hours at 24°C before putting onto the column. The void volume was 36 ml.

Symbols: ● ND activity.

Δ optical density at 280 nm.


A
B
4
3
2
1
0
0.8
0.6
0.4
0.2
0
20
60
100
NO_2^- REDUCTION (unit)
O. D. 280
ELUTION VOLUME (ml)

interpretation is possible at this time.

The ND activity is sensitive to changes in temperature (Table 10). When incubated at 60°C, the activity was stable for the first 20 minutes, and was halved by incubation for an additional 20 minutes under the same conditions.

The possible presence of a sulfhydryl group(s) in this molecule was explored by examining the effect of iodoacetate and of p-chloromercurobenzoate on the ND activity. When the supernatant of crude extracts of fully induced wild type mycelia treated with acetone was preincubated with 10^{-3}M iodoacetate for 2 hours at 0-4°C and then assayed, no inactivation of ND activity was observed. If the acetone treated preparation was preincubated with 10^{-2}M hydrosulfite, there was a loss of 30% of the original activity in the presence of 10^{-5}M iodoacetate, and a loss of 70% of the original activity in the presence of 10^{-2}M iodoacetate. Preincubation of ND with 10^{-2}M hydrosulfite and 2×10^{-5}M p-chloromercurobenzoate for 2 hours at 0-4°C resulted in a loss of 60% of the original activity (Table 11).

The possible involvement of a metal in the ND activity was studied by observing the effect of metal-complexing agents on the ND activity. Preincubation of the supernatant of an extract of fully induced wild type mycelia with cyanide, o-phenanthroline or 2,2'-

Table 10

Thermal Stability of Nitrite Reducing Activity in Acetone-Extracted Preparations of Fully Induced Wild Type Mycelia

Time of incubation (mins.)	Nitrite reduction (units/0.4 ml)
0	9.0
10	9.0
20	8.5
30	6.2
40	4.5

Ten ml of an acetone-extracted preparation of fully induced wild type mycelia were heated in a water bath at 60°C. Samples of 0.4 ml were withdrawn after 0,10,20,30 and 40 minutes of incubation and the nitrite reducing activity assayed. The results are representative of three experiments.

Table 11

Effect of Iodoacetate and p-chloromercurobenzoate on Nitrite Reducing Activity

Expt. No.	Additions to incubation mixture	Concentration	Nitrite reduction (unit)	
			-hydrosulfite	+ hydrosulfite
1	None	-	13.5 (100)	21.6 (100)
	Iodoacetate	10^{-5} M	14.7 (110)	13.5 (63)
		10^{-4} M	15.9 (118)	14.7 (68)
		10^{-3} M	11.4 (84)	12.3 (57)
		10^{-2} M	11.4 (84)	7.8 (36)
2	None	-		11.5 (100)
	p-chloro-mercurobenzoate	2×10^{-6} M		6.7 (58)
		2×10^{-5} M		4.4 (38)
		2×10^{-4} M		1.7 (15)

Samples of acetone extracted preparations of fully induced wild type mycelia were incubated for 2 hours at 0-4°C with different concentrations of iodoacetate or p-chloromercurobenzoate in the presence or absence of 10^{-2} M hydrosulfite. The nitrite reducing activity of 0.5 ml of each of the incubation mixtures was then assayed. The results are representative of four experiments. Figures in parentheses indicate the percent of the control.

bipyridine, all at 10^{-3}M for 2 hours at 0-4°C, resulted in the loss of 5,55 or 30% of the original ND activity, respectively. In the presence of 10^{-2}M hydrosulfite and an otherwise identical preincubation, cyanide and o-phenanthroline caused complete inactivation, and 2,2'-bipyridine caused a loss of 60% of the original ND activity (Table 12).

The results suggest that a sulfhydryl group(s) and a metal(s) may reside in the molecule carrying the ND activity and that these may be more exposed or reactive when the ND molecule is reduced than when it is oxidized, possibly due to a difference in conformation between the ND molecule in the oxidized and in the reduced state.

Of the four metal salts present in the trace element solution of the *Neurospora* culture medium used in our experiments, only $FeCl_3$ caused a substantial disappearance of nitrite when added to the enzyme assay mixture. $CuCl_2$, $ZnCl_2$ and Na_2MoO_4 had a negligible effect (Table 13). The amount of the metal salts used in these experiments (between 0.2 to 1.0 mg per 3 ml assay) was relatively high. The concentration of trace elements in the culture medium is between 0.037 to 4.2 µg per ml. If the mycelia could concentrate all the salts available in the medium into the cells, and all of the $FeCl_3$ thus concentrated were to be fractionated into the supernatant of the crude extracts treated with acetone, there would be 8 µg $FeCl_3$ per ml in the ND

Table 12

Effect of Metal Complexing Agents

on Nitrite Reducing Activity

Expt. No.	Addition to incubation mixture	Concentration	Nitrite reduction (unit)	
			-hydrosulfite	+ hydrosulfite
1	None		18.0 (100	23.7 (100)
	2,-2'-bipyridine	10^{-5} M	15.9 (88)	17.1 (72)
		10^{-4} M	13.5 (75)	13.5 (57)
		10^{-3} M	12.3 (68)	9.0 (38)
2	None	-	17.7 (100)	21.3 (100)
	cyanide	10^{-5} M	15.9 (90)	19.2 (90)
		10^{-4} M	16.8 (95)	13.5 (44)
		10^{-3} M	16.8 (95)	0 (0)
3	None		18.0 (100)	23.7 (100)
	o-phenanthroline	10^{-5} M	6.9 (38)	9.0 (38)
		10^{-4} M	7.8 (43)	9.0 (38)
		10^{-3} M	7.8 (43)	0 (0)

Samples of acetone extracted preparations of fully induced wild type mycelia were incubated for 2 hours at 0-4°C with different concentrations of 2,-2'-bipyridine, cyanide and o-phenanthroline in the presence or absence of 10^{-} M hydrosulfite. The nitrite reducing activity of 0.5 ml of each of the incubation mixtures was then assayed. The results are representative of four experiments. Figures in parentheses indicate the percent of the control.

Table 13

Nitrite Reducing Activity of Some Trace Elements Present in the Culture Medium

Trace element solution (w/v)	Nitrite reduction (units)
$CuCl_2$ (0.1%)	1.1
$FeCl_3$ (0.3%)	80.0
Na_2MoO_4 (0.1%)	3.5
$ZnCl_2$ (0.5%)	2.0

Two tenths of a millilitre of trace element solution was used in place of acetone-extracted preparation of mycelia in a standard assay. The disappearance of nitrite from the assay mixture was determined. The incubation was for 20 minutes at 30°C. The results shown are the average of three determinations which were within 20% of each other.

containing preparation. The quantity of $FeCl_3$ added to a typical ND assay mixture in the form of ND containing extract, if all the above assumptions were correct, would be two orders of magnitude less than the concentration of $FeCl_3$ in the assay mixtures giving rise to the results displayed in Table 13. Furthermore, the presence of 30 µg of $FeCl_3$ in the assay mixture did not cause any disappearance of nitrite. For this reason, it would seem unlikely that $FeCl_3$ from the trace element solution was responsible for the disappearance of nitrite in the assay of ND activity.

5. The physiologically important activity. The above results show that the ND activity differs from the AP activity in the following ways: ND, unlike AP does not catalyze the production of ammonia from the reduction of nitrite; ND, unlike AP is filterable and is not precipitable by acetone.

The physiologically important nitrite reductase activity should be present in strains which can utilize nitrite (or nitrate) as a source of nitrogen, and should be absent from strains which cannot utilize nitrite; a physiologically unimportant activity, on the other hand, could be present in all strains regardless of their ability to use nitrite as a source of nitrogen. AP activity was present in preparations of the wild type strain, and in those of *nit-1* and *nit-3* when the mycelia were exposed to nitrite or nitrate ions; it was absent from extracts of strains *nit-2*, *nit-4*, *nit-5* and *nit-6*

regardless of the nitrogen source to which the mycelia were exposed. The ND activity is found in extracts of all strains of *Neurospora* exposed to any of the nitrogen sources listed in Table 14. Only the wild type strain, *nit-1* and *nit-3* contain AP and ND activities and utilize nitrite, whereas strains *nit-2*, *nit-4*, *nit-5* and *nit-6* which cannot utilize nitrite as nitrogen source, contain ND but not AP activity. Consequently, the ND activity by itself cannot support growth on nitrite. Therefore, AP is the most likely candidate for the physiologically important activity.

6. Summary. Our results show that AP activity and ND activity are present in crude extracts of wild type mycelia exposed to nitrate or nitrite. The AP activity is believed to be physiologically important in the assimilation of nitrite. In later sections, the AP activity will be designated as the nitrite reductase activity.

B. Regulation of Nitrite Reductase - Induction

It has been shown in many systems that nitrite reductase is inducible by nitrite and nitrate (Table 2). Results in Table 14 show that nitrite reductase (AP activity) is present only when mycelia are exposed to a medium containing nitrite or nitrate. Protein synthesis

Table 14

Nitrate Reductase, Nitrite Reducing and Ammonia Producing Activities in Wild Type and Nitrate-non-utilizing Strains of *Neurospora* Exposed to Different Nitrogen Sources

Strain	Ability to grow on as sole source of nitrogen			Nitrogen source in "induction" medium	Specific activity (units/mg of protein)			
					crude extract			Acetone extracted preparation
	NO_3^-	NO_2^-	NH_4^+		NADPH-NR	ND	AP	ND
wild type	+	+	+	NO_2^-	2.9	13.4	9.2	3.4
				NO_3^-	10.3	14.6	8.9	4.2
				NH_4^+	0	4.5	0.5	2.7
				-N	0	7.1	0	2.7
nit-1A	-	±	+	NO_3^-	0	5.6	2.3	2.9
				NH_4^+	0	6.1	0	2.9
				-N	0	5.5	2.7	3.1
nit-2a	-	-	+	NO_3^-	0	5.4	<0.5	3.1
				NH_4^+	0	3.8	<0.5	2.8
				-N	0	3.6	0	2.8
nit-3a	-	±	+	NO_3^-	0	6.0	2.7	2.1
				NH_4^+	0	3.8	0	2.4
				-N	0	4.7	0	2.8
nit-4a	-	-	+	NO_3^-	0	4.3	0	2.5
				NH_4^+	0	3.7	0	2.7
				-N	0	3.7	<0.9	2.7
nit-5A	-	-	+	NO_3^-	0	3.4	<0.5	1.7
				NH_4^+	0	2.9	0	2.0
				-N	0	2.7	0	1.9
nit-6A	-	-	+	NO_3^-	8.2	5.2	<0.5	3.3
				NH_4^+	0	3.2	0	2.9
				-N	0	4.9	0	3.7

is required for the induced formation of the enzyme. During a three hour induction of nitrite reductase in wild type mycelia by nitrate, the presence of 1 µg/ml of cycloheximide in the induction medium reduced the appearance of AP activity by 80% (2.1 units/mg of protein as compared to 9.5 units/mg of protein in its absence). In some systems the role of nitrate in the induction has been found to be a direct one such as in *Azotobacter*. In systems like *Anabaena* and radish cotyledons, nitrite reductase was described as indirectly induced by nitrate. The question we are attempting to answer in this section is: Does nitrate induce nitrite reductase directly in *Neurospora* or must it first be converted to nitrite? The following approaches have been used in an attempt to obtain an answer: 1) The comparison of the kinetics of induction of nitrate and nitrite reductases by nitrate; 2) The induction of nitrite reductase by nitrate in the presence of tungstate; 3) The induction of nitrite reductase by nitrate in strains which cannot utilize nitrate; 4) The induction of nitrite reductase by nitrate in the presence of ammonium ions at a concentration which represses nitrate reductase.

1. <u>Nitrate as a direct inducer of nitrite reductase</u>.

a. Kinetics of induction of nitrate reductase and of nitrite reductase by nitrate. If nitrate has to be reduced to nitrite in order to induce nitrite reductase, then one would expect that nitrate reductase would begin

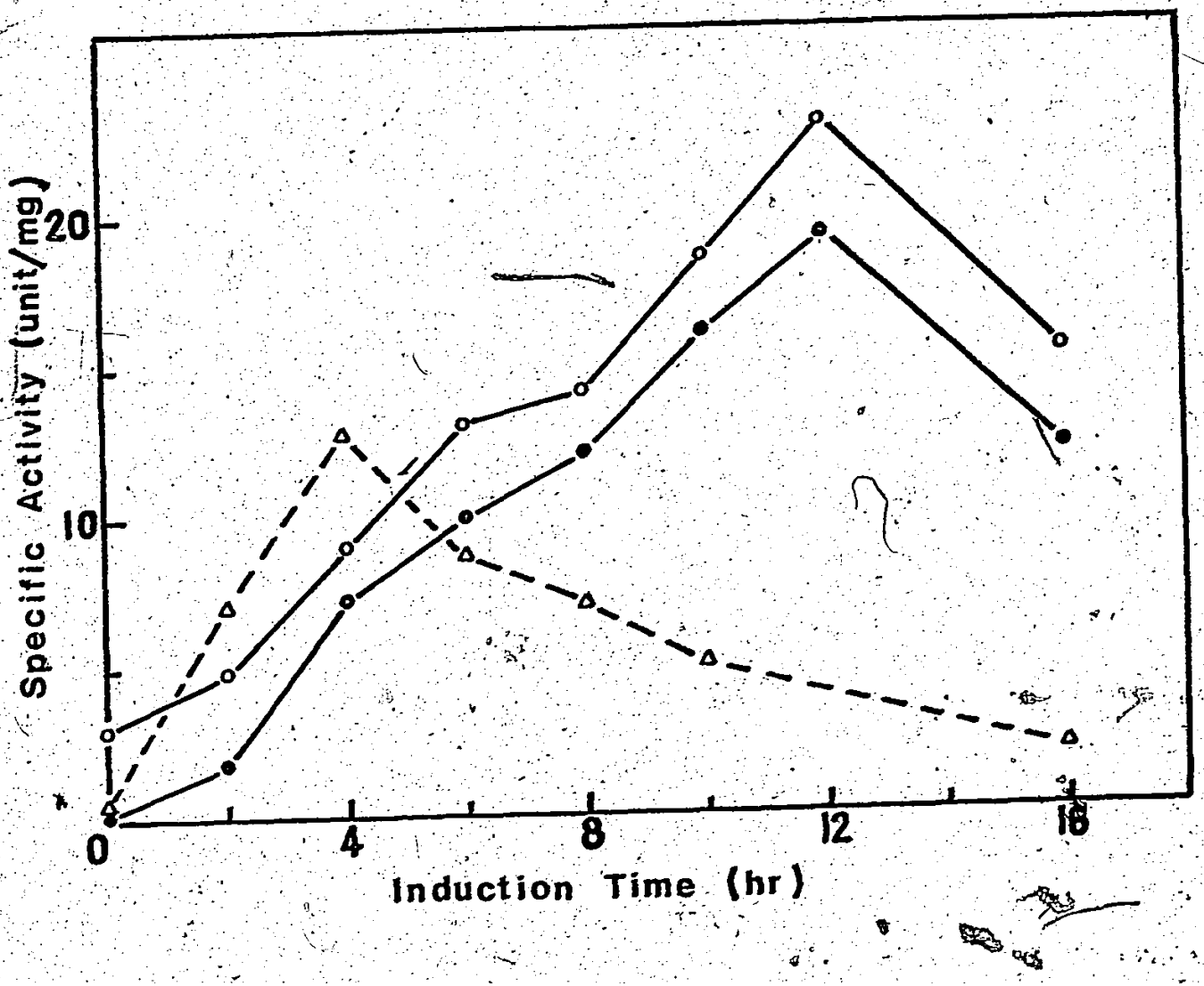
Figure 7. Kinetics of induction of NADPH-NR and nitrite reductase in wild type mycelia exposed to 20 mM nitrate medium.

Wild type mycelia grown in ammonia medium as described in Table 15 were induced for different lengths of time in basic medium containing 20 mM nitrate. The mycelia were then harvested, extracted and were assayed for NADPH-nitrate reductase and nitrite reductase activities as described in Methods. The result is a representation of six experiments.

Symbols: Δ NADPH-NR activity.

o nitrite reduction.

● ammonia production.


Specific Activity (unit/mg)
20
10
0
4
8
12
16
Induction Time (hr)

to appear earlier than nitrite reductase following exposure of the mycelia to nitrate. As shown in Figure 7, nitrite reductase and nitrate reductase activities in *Neurospora* began to appear almost at the same time in response to exposure to nitrate. Nitrite reductase reached its peak specific activity after 10-12 hours of induction, while nitrate reductase reached it after 3-4 hours. Extrapolation of the curves representing the time-dependent accumulation of the two enzymes to zero specific activity indicates that both enzymes were formed within an hour of exposure to nitrate in the culture medium (Figure 8). When nitrite is used as the inducer, nitrite reductase also appears after 2 hours and reaches its peak at 6-8 hours of induction (Figure 9). The simultaneous appearance of nitrate and nitrite reductases suggests that nitrate acts directly as an inducer of nitrite reductase. This observation confirms a similar one made by Garrett (16).

b. Induction of nitrite reductase by nitrate in the presence of tungstate. Tungstate, when present in the nitrate induction medium has been reported to prevent the formation of active nitrate reductase in *Neurospora* (66) and in cultured tobacco cells (20). Under these conditions, nitrate will not be reduced to nitrite. If nitrate has to be reduced to nitrite before it can induce nitrite reductase, then the presence of tungstate should prevent the induction of nitrite reductase. Normal

Figure 8. Kinetics of induction of NADPH-nitrate reductase and nitrite reductase during the first five hours of exposure to 20 mM nitrate.

Wild type mycelia grown on ammonia medium as described in Table 15 were induced for different lengths of time in nitrate medium. The mycelia were then harvested, extracted and were assayed for NADPH-nitrate reductase and nitrite reductase (AP) activities as described in Methods. The result is representative of three experiments.

Symbols: Δ NADPH-NR activity.

o NiR (AP) activity.

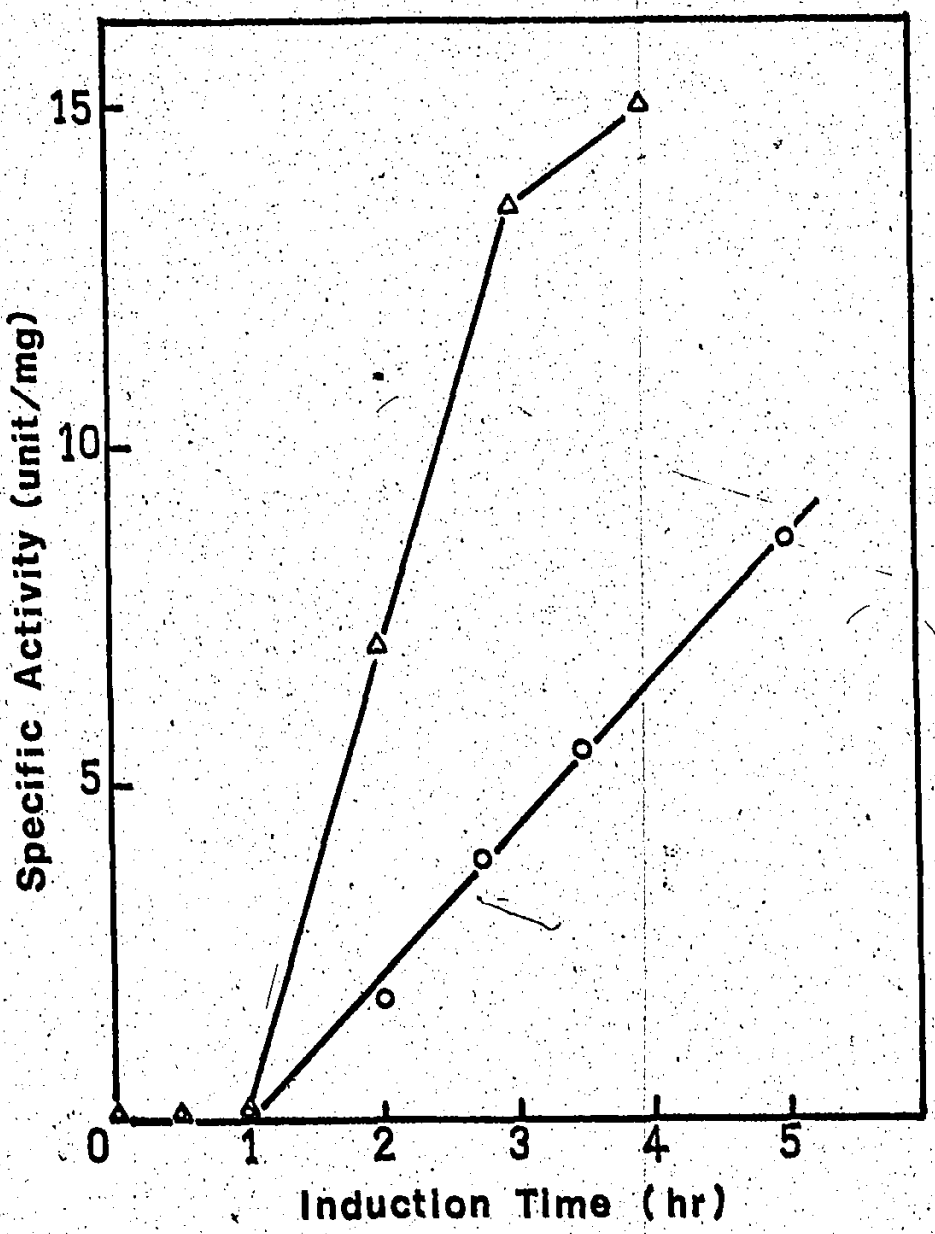
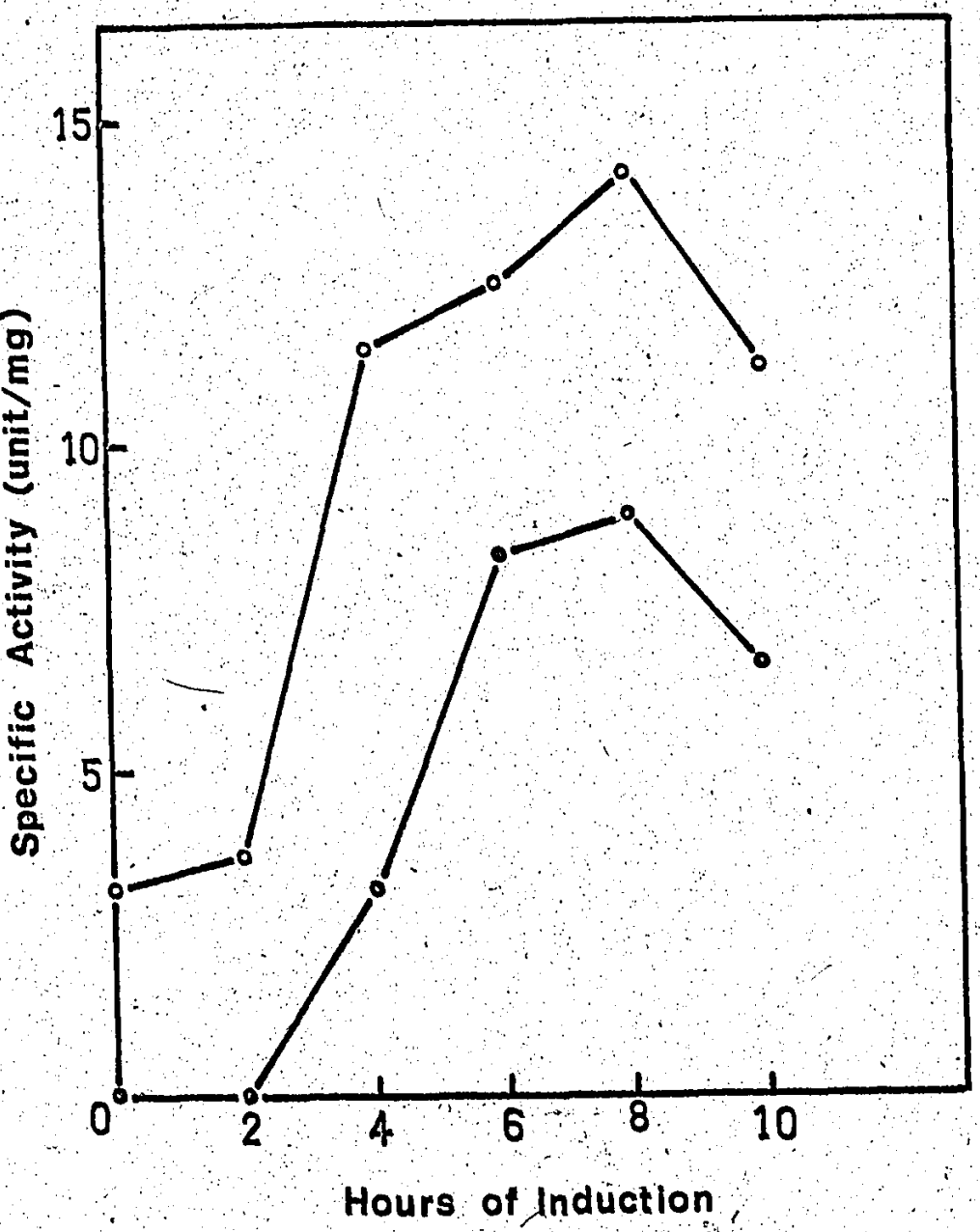

15
10
5
0
1
2
3
4
5
Specific Activity (unit/mg)
Induction Time (hr)

Figure 9. Effect of the time of exposure of wild type mycelia to 10 mM nitrite on the formation of nitrite reductase.

Wild type mycelia grown on ammonia medium as described in Table 15 were exposed to 10 mM nitrite for different lengths of time. The mycelia were then harvested, extracted and were assayed for nitrite reductase activity. The result shown is representative of six experiments.

Symbols: o nitrite reduction.

● ammonia production.


15
10
5
0
Specific Activity (unit/mg)
2
4
6
8
10
Hours of Induction

levels of nitrite reductase activity were induced by nitrate in the presence of 6 mM tungstate (Table 15). The induction kinetics are similar to those when nitrite reductase is induced by nitrate alone, except that the maximum activity attained by induction is lowered by the tungstate treatment (Figure 10). There is a large increase in nitrite reduction after 24 hours of induction in tungstate-containing medium. This is due to an increase in the ND activity. Tungstate may stimulate the formation of the ND activity.

c. Induction of nitrite reductase by nitrate in nitrate non-utilizing strains. Nitrate non-utilizing strains which do not have normal NADPH-nitrate reductase activity cannot reduce nitrate to nitrite (3,61). If nitrite reductase could be induced in these strains by nitrate, it would suggest that nitrate is a direct inducer of the enzyme. When *nit-1*, *nit-2*, *nit-3*, *nit-4* and *nit-5* mycelia were exposed to nitrate for 8 hours, only extracts of *nit-1* and *nit-3* mycelia catalyzed the production of ammonia from nitrite (Table 16). The nitrite reducing (AP) activity extracted from these mutants was quite low when compared to that prepared from wild type mycelia induced in the same way. The absence of nitrite reductase activity in *nit-2*, *nit-4* and *nit-5* mycelia may be because the mutations in these strains are involved in the regulation of both nitrate and nitrite

Table 15

The Effect of Nitrite, Nitrate and Nitrate Plus Tungstate on the Induction of Nitrate and Nitrite Reductases in Wild Type Mycelia

Nitrogen source in induction medium	Specific activity (units/mg protein)		
	NADPH-NR	nitrite reduction	ammonia production
NO_2^- (10 mM)		14.1	8.9
NO_3^- (20 mM)	14.9	24.8	19.3
NO_3^- (20 mM) + $WO_4^=$ (6 mM)	0.4	19.3	13.6

Wild type mycelia pads, progrown in ammonia medium for 72 hours at 27°C without shaking, were exposed to basic media containing different nitrogen sources for 3.5 hours when nitrate reductase activity was to be measured, and for 10 hours when nitrite reductase activity was to be examined, in a shaker at 27°C. The mycelia were harvested, extracted and assayed for nitrate and nitrite reductase activities as described in Methods. The results shown are representative of three experiments.

Figure 10. The effect of the time of exposure of wild type mycelia to nitrate medium containing no tungstate or 6 mM tungstate on the induction of nitrite reductase.

Mycelia grown on ammonia medium as described in Table 15, were induced for different lengths of time in nitrate media containing 20 mM nitrate and 6 mM sodium tungstate (Broken line) or no tungstate (solid line). The mycelia were harvested, extracted and assayed as described in Methods. The results shown are representative of three experiments.

Symbols: Δ,○ nitrite reduction.

▲,● ammonia production.

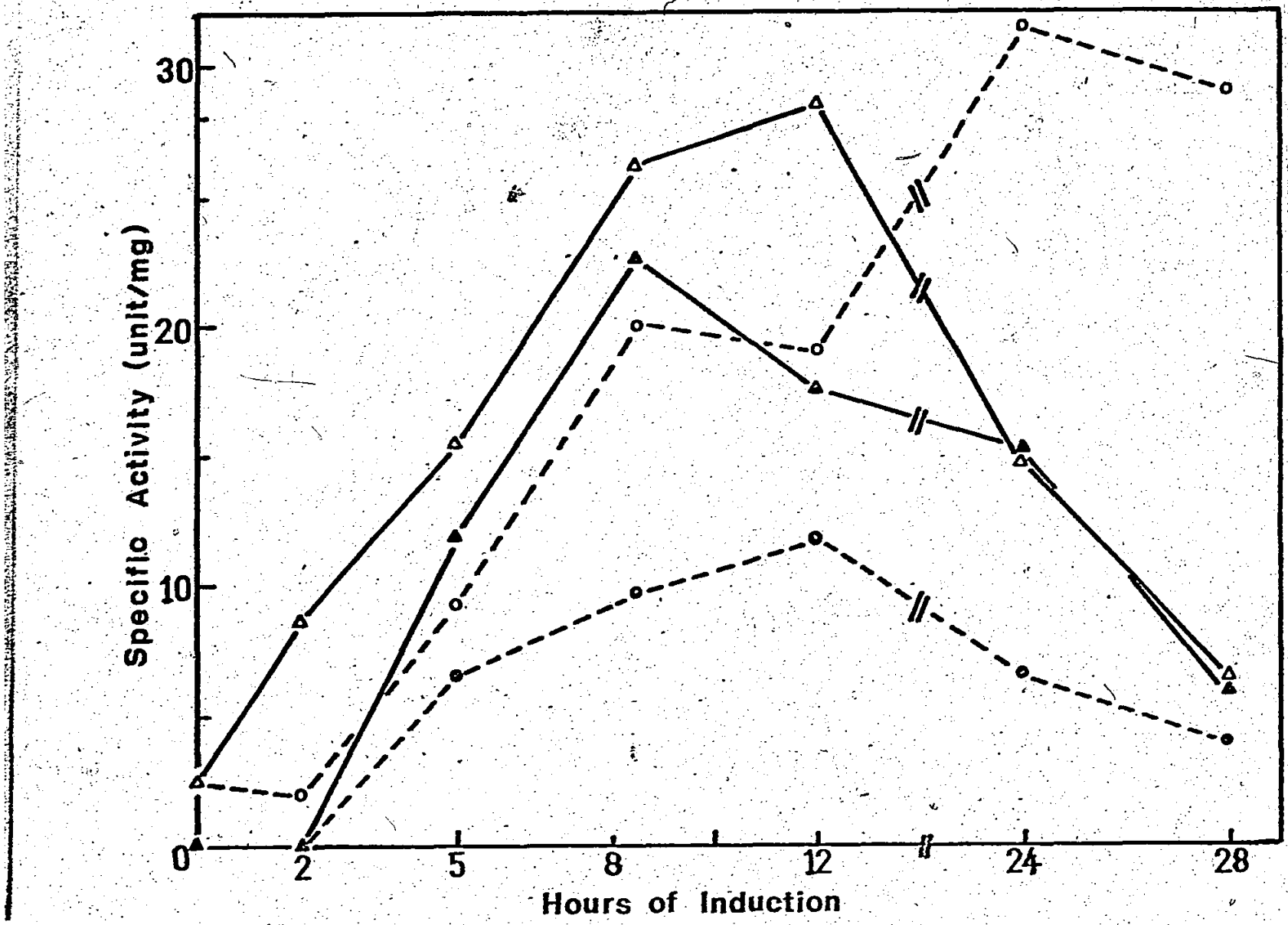

Specific Activity (unit/mg)
30
20
10
0
2
5
8
12
24
28
Hours of Induction

Table 16

The Effect of Nitrate on the Induction of Nitrite Reductase in Wild Type and Nitrate Non-Utilizing (*nit*) Strains

Strains	Specific activity (units/mg protein)	
	nitrite reduction	ammonia production
Wild type	14.6	9.7
nit-7A	5.6	2.3
nit-8a	5.4	<0.5
nit-3a	6.0	2.7
nit-1a	4.3	0
nit-6A	3.4	<0.5

Mycelia of different strains, pregrown in ammonia media as described in Table 15, were induced in 20 mM nitrate media for 8 hours. The mycelia were harvested, extracted and assayed for nitrite reductase activity. The results are representative of five experiments.

reductases. The loci affected in strains *nit-1* and *nit-3* are believed to be involved in the control of the structure of nitrate reductase, because each strain contains a partial nitrate reductase activity (61).

d. Induction of nitrite reductase by nitrate in the presence of ammonium ions. Ammonium ions have been shown to inhibit the induction of nitrite reductase by nitrate (Table 2). In *Neurospora*, the induction of nitrite reductase by nitrate is partially repressed by ammonium ions. The extent of the repression depends on the concentration of ammonium ions present in the nitrate induction medium (Table 17). Ammonium ions have also been shown to repress the formation of nitrate reductase in *Neurospora* (67). If the presence of ammonium ions in the nitrate medium caused a complete repression of nitrate reductase whilst nitrite reductase was only partially repressed, the result would suggest that the latter is directly induced by nitrate because no nitrite is produced from nitrate in the absence of nitrate reductase.

Neurospora mycelia grown in ammonia medium were exposed to nitrate (20 mM) plus different concentrations of ammonium tartrate (0-40 mM). Mycelia were harvested after 3 1/2 hours for nitrate reductase determinations and after 9 hours for measurements of nitrite reductase. The effect of different concentrations of ammonium ions

Table 17

The Effect of Ammonium Ions in Nitrate Medium on the Induction of Nitrite Reductase in Wild Type Mycelia

Expt. No.	Nitrogen sources in induction medium	Specific activity (units/mg protein)	
		nitrite reduction	ammonia production
1	NO_3^- (20 mM)	17.9	15.1 (100)
	NO_3^- (20 mM) + NH_4^+ (2 mM)	16.2	13.8 (91)
	NO_3^- (20 mM) + NH_4^+ (4 mM)	14.0	11.5 (76)
	NO_3^- (20 mM) + NH_4^+ (8 mM)	13.8	10.1 (67)
	NO_3^- (20 mM) + NH_4^+ (15 mM)	11.7	9.5 (63)
	NO_3^- (20 mM) + NH_4^+ (30 mM)	10.5	8.2 (54)
2	NO_3^- (20 mM)	12.2	0.0
	NO_3^- (20 mM) + NH_4^+ (40 mM)	7.7	5.1

Mycelia grown on ammonia medium (1) or on CA medium (2) as in Table 15 were induced for 10 hours in 20 mM nitrate media containing different concentrations of ammonium tartrate. The mycelia were harvested, extracted and assayed for nitrite reductase activity. The results are representative of three experiments.

in the culture medium on the induction of nitrate and nitrite reductase is shown in Table 18. The induction of both enzymes is affected by ammonium ions but the repression of nitrate reductase by ammonium ions is stronger than that of nitrite reductase. At 40 mM ammonium ions, the induction of nitrate reductase was repressed by 90% whereas that of nitrite reductase was reduced by only 50%. When ammonium ions are present in the nitrate induction medium at a concentration of 70 mM, one would predict that nitrate reductase would be completely repressed (Figure 11), but nitrite reductase would not. There would be 7.2 units of nitrite reductase activity per mg protein left, or approximately 50% of the maximum induced specific activity. As shown in part B of Table 18, the presence of 100 mM ammonium tartrate in the nitrate medium virtually blocked the induced formation of nitrate reductase whereas 35% of the induced level of nitrite reductase was still formed under these conditions. The result showed that nitrite reductase was synthesized under the conditions where virtually no nitrite was being produced from nitrate.

The results shown thus far suggest that nitrate is a direct inducer of nitrite reductase. It should be noted here that residual nitrate reductase is present in wild type mycelia induced in nitrate medium containing 6 mM tungstate (Table 15) and perhaps trace amounts of

Table 18

The Effect of the Concentration of Ammonium Ions in Nitrate Medium on the Induction of NADPH-nitrate Reductase and Nitrite Reductase

Expt. No.	Nitrogen sources in induction medium	Specific activity (units/mg protein)		
		NADPH-NR	nitrite reduction	ammonia production
A	NO_3^- (20 mM)	21.6	18.2	15.5
	NO_3^- (20 mM) + NH_4^+ (5 mM)	7.4	14.2	11.2
	NO_3^- (20 mM) + NH_4^+ (10 mM)	6.8	13.0	9.6
	NO_3^- (20 mM) + NH_4^+ (20 mM)	3.5	12.5	9.2
	NO_3^- (20 mM) + NH_4^+ (40 mM)	2.0	10.9	7.6
B	NO_3^- (20 mM)	18.1	20.9	16.4
	NO_3^- (20 mM) + NH_4^+ (100 mM)	0.24	7.5	5.6

Wild type mycelia grown on ammonia medium as in Table 15, were induced in 20 mM nitrate media containing different concentrations of ammonium ions for 3.5 hours when nitrate reductase activity was to be measured, and for 9 hours when nitrite reductase activity was to be determined. The mycelia were harvested, extracted and assayed as described in Methods. The results shown are representative of three experiments.

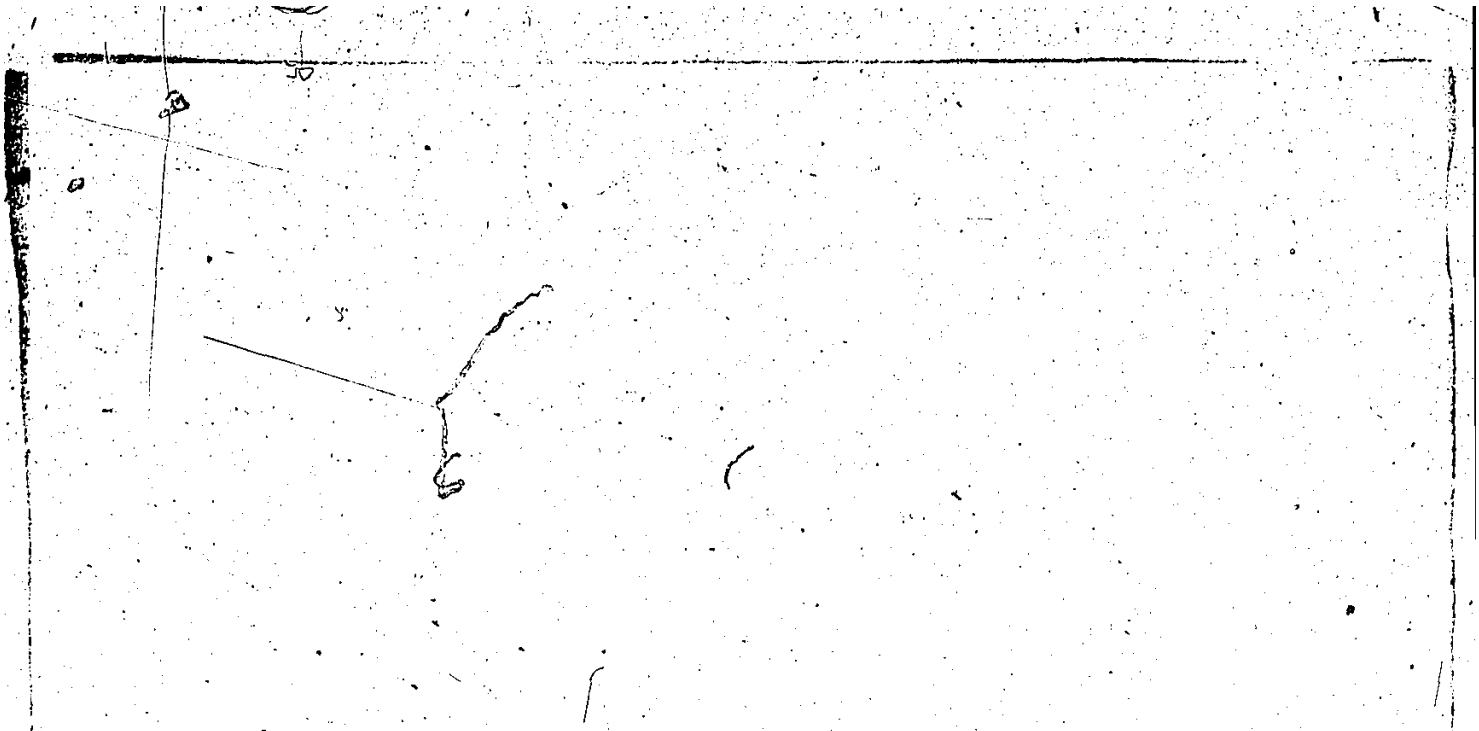
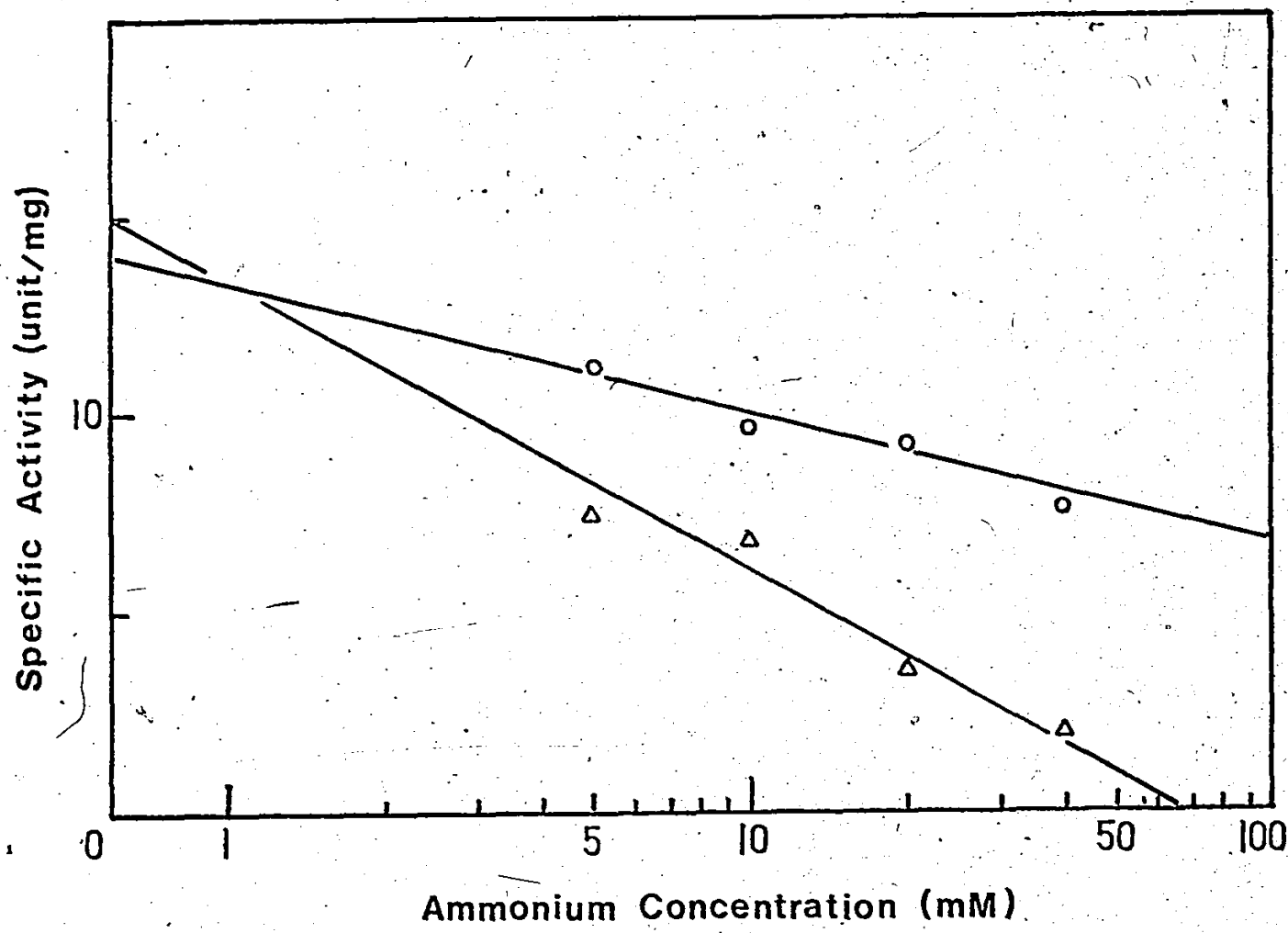


Figure 11. Effect of the concentration of ammonium ions in nitrate media on the induction of NADPH-nitrate reductase and nitrite reductase in wild type mycelia.

Mycelia grown on ammonia medium as described in Table 15, were induced for 3.5 hr (for NADPH-NR determination) and 9 hours (for NiR measurement), respectively, in basic medium containing 20 mM nitrate plus different concentrations of ammonium tartrate. The mycelia were harvested, extracted and assayed as described in Methods. The results shown are representative of three experiments.

Symbols: Δ NADPH-NR activity.

o NiR (AP) activity.


Specific Activity (unit/mg)
10
0
1
5
10
50
100
Ammonium Concentration (mM)

the enzyme may be present in *nit-1* and *nit-3* when these are induced by nitrate. If the trace amounts of nitrite produced from nitrate by this residual nitrate reductase were responsible for the appearance of nitrite reductase activity, then very low levels of nitrite should be sufficient to induce nitrite reductase.

A threshold concentration of 2 mM nitrite in the culture medium is required for the appearance of nitrite reductase (Table 19). This observation suggests (qualitatively) that trace amounts of nitrite may not be effective in the induction of nitrite reductase.

The quantity of nitrite produced by the trace amounts of nitrate reductase in the presence of tungstate was determined in *nit-6*, a strain which does not utilize nitrite, and in which any nitrite produced will accumulate in the cell. Extracts of *nit-6* mycelia which had been exposed to 10 mM nitrate plus tungstate contained 3.2 nmoles of nitrite per mg of extract protein. This intracellular concentration of nitrite is lower than that of wild type mycelia exposed to 2 mM nitrite (*i.e.*, 6.4 nmole per mg of extract protein) (Table 20). Exposure of mycelia to at least 2 mM nitrite, was required for the induced appearance of nitrite reductase, and resulted in an intracellular accumulation of 6.4 nmoles nitrite per mg of extract protein. It would appear, consequently, that 3.2 nmoles nitrite per mg of extract protein cannot effectively induce nitrite reductase, unless there is

Table 19

The Effect of Different Concentrations of Nitrite on the Induction of Nitrite Reductase in Wild Type Mycelia

Concentration of NO_2^- in induction medium (mM)	Specific activity (units/mg protein)	
	nitrite reduction	ammonia production
0	7.5	0
1	7.8	0
2	8.2	1.8
5	7.8	4.1
10	8.9	5.7
15	6.5	2.9

Mycelia grown on ammonia medium, as in Table 15, were induced for 10 hours in neutralized basic media containing different concentrations of nitrite. The mycelia were harvested, extracted and assayed for nitrite reductase activity. The results are representative of four experiments.

Table 20

Concentration of Nitrite in Extracts of Wild Type and *nit-6* Mycelia Exposed to Different Nitrogen Sources

Strain	Nitrogen sources in incubation medium (concentration)	Total nitrite content (nmole/mycelia)*	Specific nitrite content (nmole/mg of protein)
Wild type	NO_2^- (2 mM)	275	6.4
	NO_2^- (5 mM)	425	12.2
	NO_3^- (10 mM)	20	0.8
nit-6A	NO_3^- (10 mM) + $WO_4^=$ (6 mM)	105	3.2

* nmole of nitrite present in extract made from one mycelial pad.

Wild type and *nit-6* mycelial pads grown on ammonium medium as in Table 15, were exposed to basic medium containing different nitrogen sources for 3.5 hours. The mycelia were harvested and extracted. The concentration of nitrite in the mycelial extracts was determined as described in Methods. The results shown are representative of three experiments.

compartmentation of the nitrite pool in the mycelia.

The nitrite reductase activity present in *nit-1* and *nit-3* mycelia induced on nitrite and on nitrate was compared, and is shown in Table 21. No difference in the level of nitrite reductase activity present in extracts of *nit-1* and *nit-3* mycelia induced under the two conditions was observed. When the nitrate induction medium was neutralized with sodium bicarbonate, as is the customary procedure to avoid nitrite toxicity, the above pattern was unchanged.

2. Role of nitrate reductase in the induction of nitrite reductase. The presence of nitrite reductase in wild type mycelia induced by exposure to nitrate plus tungstate, or in *nit-1* and *nit-3* mycelia induced by exposure to nitrate suggests that active (normal) NADPH-nitrate reductase, which is absent under the above conditions, is not essential for the induction of nitrite reductase. The effect of different concentrations of ammonium ions on the induction of nitrate and nitrite reductases (Table 18 and Figure 9) further suggests that NADPH-nitrate reductase (active or inactive) may not be needed at all for the induction of nitrite reductase by nitrate.

One explanation for the presence of nitrite reductase activity in wild type mycelia induced in tungstate-containing nitrate medium, or in *nit-1* and *nit-3* mycelia

Table 21

Effect of Different Nitrogen Sources on the Induction of Nitrite Reductase in *nit-1a* and *nit-3a* Mycelia

Strains	Nitrogen source in induction medium	Specific activity (units/mg)	
		nitrite reduction	ammonia production
nit-1a	NO_2^- (10 mM)*	5.4	2.5
	NO_3^- (20 mM)	5.6	2.3
nit-3a	NO_2^- (10 mM)*	3.7	2.0
	NO_3^- (20 mM)	5.8	2.1
	NO_3^- (20 mM)*	4.5	1.9

Mycelia grown on ammonia medium as described in Table 15 were induced for 10 hours in basic media containing different nitrogen sources. The mycelia were harvested, extracted and assayed for nitrite reductase activity. The results are representative of five experiments.

* Media neutralized with sodium bicarbonate.

induced by nitrate, would be that inactive or partial nitrate reductase may be effective as an inducer of nitrite reductase. Strains *nit-1* and *nit-3* have been shown to have derepressible - *i.e.*, ammonia repressible, but not obligately inducible by nitrate - partial nitrate reductase activities (NADPH-cytochrome-c-reductase in the case of strain *nit-1*, and benzyl viologen-nitrate reductase in the case of strain *nit-3*) (61). If nitrite reductase were formed in mycelia of these strains when they were derepressed on a medium containing no nitrogen source, this would suggest that a derepressible partial nitrate reductase can help to induce nitrite reductase. When *nit-1* and *nit-3* mycelia were exposed to no nitrogen medium for different lengths of time, extracts of the mycelia catalyzed the production of ammonia from nitrite (Table 22). The AP activity observed in extracts of *nit-1* mycelia was higher than that in *nit-3* mycelia under the above conditions. The activity also increased with the length of derepression. These results suggest that AP activity can be induced in medium containing no nitrogen source if a partial nitrate reductase is present.

3. Summary. The observations reported suggest that nitrate is a direct inducer of nitrite reductase. Nitrate reductase is not essential for the induction of nitrite reductase, but inactive or partial nitrate reductase by

Table 22

The Derepression of Nitrite Reductase in *nit-1a* and *nit-3a* Mycelia in Media Containing No Nitrogen Source

Strains	Duration of exposure (hours)	Specific activity (units/mg protein)	
		nitrite reduction	ammonia production
nit-1a	2	4.5	0.8
	4	4.1	0.8
	6	5.9	1.1
	8	6.5	2.7
nit-3a	2	3.0	0.6
	4	4.4	1.3
	6	2.2	0.9
	8	4.6	0.8

Mycelia grown on ammonia medium as described in Table 15 were exposed to basic medium containing no nitrogen source. The mycelia were harvested at different time intervals as listed, extracted and assayed for nitrite reductase activity. The results are representative of three experiments.

itself may be involved in the induction of nitrite reductase, though weakly.

C. Regulation of Nitrite Reductase - Repression

Ammonium ions have been shown to repress nitrite reductase in many systems. In *Neurospora*, the physiologically important nitrite reductase activity is absent from extracts of wild type mycelia exposed to ammonia (Table 3). When ammonium ions are present in nitrate induction medium, nitrite reductase (AP) was partially repressed (Table 17). The extent of repression depended on the concentration of ammonium ions present.

1. Role of ammonium ions in the repression of nitrite reductase. Little is known about the role of ammonium ions in the repression of nitrite reductase. Ammonium ions may be acting in the following way: inhibition of nitrite reductase activity *in vivo*, promotion of the production of inhibitors of nitrite reductase, or control of the synthesis of the enzyme. Some of these possibilities are examined below.

When ammonium ions were present in the nitrite reductase assay mixture at a concentration of 3 mM, no inhibition of nitrite reductase activity was observed (Table 23). This observation agrees with previous ones made by Mulkins with desalted extracts of *Neurospora* (40).

Table 23

Effect of Ammonium Ions in the Assay Mixture on Nitrite Reducing Activity

Nitrogen sources in "induction" medium	Nitrite reduction (units/mg protein) additions to enzyme assay mixture	
	none	3 mM NH_4Cl
NO_3^- (10 mM)	17.7	18.2
NH_4^+ (10 mM)	10.1	9.8
No nitrogen source	10.0	11.2

Wild type mycelia pregrown on ammonia medium for 72 hours in stationary culture at 27°C, were incubated for 10 hours at 27° with shaking in basic medium containing different nitrogen sources. The mycelia were harvested, extracted and assayed for nitrite reduction in the presence and absence of 3 mM ammonium chloride. The results shown are representative of six experiments. AP activity was not measured due to the presence of ammonium chloride in the assay mixture.

Due to the high concentration of ammonium ions used, it was impossible to measure the production of ammonia in the assay, consequently the disappearance of nitrite was determined. It was assumed that a decrease in the AP activity would be accompanied by a corresponding decrease in the disappearance of nitrite.

The possibility that inhibitors of nitrite reductase activity were present in mycelia exposed to ammonium ions was studied as follows: Extracts of wild type mycelia incubated for 10 hours in ammonia medium and of fully induced wild type mycelia were mixed and incubated on ice for one hour. The nitrite reductase activity of the resultant mixture was the sum of nitrite reductase activities exhibited by the two different extracts when assayed separately (Table 24). This observation suggests that inhibitors of nitrite reductase are not produced in mycelia exposed to ammonium ions.

From the above results, it would appear that the inhibitory effect of ammonium ions on nitrite reductase *in vivo* is not due to an inhibition of the nitrite reductase activity, but to inhibition of the synthesis or processing of the enzyme.

Ammonium ions may be directly involved in the repression of nitrite reductase synthesis, or indirectly involved, *i.e.*, they have to be metabolized in order to repress nitrite reductase. The above possibilities were

Table 24

Effect of the Presence of Extracts of Mycelia Exposed to Ammonia Medium in the Assay Mixture on Nitrite Reductase Activity

Mixture				Activity (units/assay)	
source	quantity ml	+ source	quantity ml	nitrite reduction	ammonia production
N	0.2			28.2	17.2
-		A	0.2	8.0	0.5
N	0.2	+ A	0.2	37.2	20.0

Wild type mycelial pads prepared as described in Table 23, were incubated for 10 hours in basic medium containing 10 mM nitrate, or 20 mM ammonia as nitrogen sources. The mycelia were harvested, and extracted. Two tenths of a millilitre of the extract of the mycelia exposed to nitrate (N) and 0.2 ml of the extract of the mycelia exposed to ammonia (A), were assayed separately and in a mixture. The protein concentration of the two extracts were 6.9 and 7.75 mg/ml, respectively. The mixture was incubated for one hour on ice before assaying for nitrite reductase activity. The results shown are representative of four experiments.

explored with the use of strain *am-1a*, a mutant lacking glutamate dehydrogenase activity (14).

The presence of ammonium ions at a concentration of 15 mM in the nitrate induction medium, caused a 40% repression of the formation of nitrite reductase in wild type mycelia, but showed no such effect in similarly treated *am-1a* mycelia (Tables 17 and 25). Extracts from *am-1a* mycelia induced in nitrate medium containing 2 mM and 4 mM ammonium ions had a relatively elevated level of nitrite reductase activity. This effect is not explicable at present. Since the *am-1a* strain does not assimilate ammonium ions, the lack of repression by ammonium ions would suggest that ammonium ions have to be metabolized to have an effect on nitrite reductase synthesis. Other possibilities, however, must first be ruled out.

The absence of repression by ammonium ions in *am-1a* mycelia is not due to the fact that *am-1a* mycelia were grown in casamino acids (CA) medium. The formation of nitrite reductase in wild type mycelia pregrown in CA medium was repressed in the same way as that in mycelia grown in ammonia medium (Table 17).

The difference between the effect of ammonium ions on the induction of nitrite reductase in wild type and in *am-1a* mycelia could be due to a difference in the transport of nitrate or ammonium ions in the two strains. Schloemer and Garrett (56) have shown that although the

Table 25

Effect of Ammonium Ions in Nitrate Media on the Induction of Nitrite Reductase in *am-1a* mycelia

Nitrogen sources in induction medium	Specific activity of extract (units/mg protein)	
	nitrite reduction	ammonia production
NO_3^- (20 mM)	23.2	19.2
NO_3^- (20 mM) + NH_4^+ (2 mM)	28.6	23.0
NO_3^- (20 mM) + NH_4^+ (4 mM)	27.1	21.1
NO_3^- (20 mM) + NH_4^+ (8 mM)	25.8	19.9
NO_3^- (20 mM) + NH_4^+ (15 mM)	25.2	20.7
NO_3^- (20 mM) + NH_4^+ (30 mM)	24.3	18.5

Mycelia pregrown on ammonia medium as described in Table 23, were induced for 10 hours at 27°C with shaking in nitrate medium containing different concentrations of ammonium ions. The mycelia were harvested, extracted and assayed as described in Methods. The results are representative of six experiments.

development of the nitrate transport system in *Neurospora* is not affected by the presence of ammonium ions in the culture medium, the uptake of nitrate by the cell is non-competitively inhibited by ammonium ions. If the uptake of nitrate by *am-1a* mycelia is not affected by ammonium ions as it is in the wild type strain, the lack of repression by ammonium ions in the *am-1a* strain may be explained as being due to an uninhibited accumulation of nitrate, which would then be available for the induction of nitrite reductase. This question was explored by determining the nitrate content of extracts of mycelia exposed to nitrate medium containing different concentrations of ammonium ions. There is little difference between the nitrate content of extracts of *am-1a* and of wild type mycelia exposed to nitrate induction media containing varying concentrations of ammonium ions (Table 26). In fact, the inhibitory effect of ammonium ions on the accumulation of nitrate is greater in *am-1a* mycelia than in wild type mycelia. The result shows that the effect of ammonium ions on the uptake of nitrate is similar in both strains.

Another alternative explanation for the lack of repression of nitrite reductase by ammonium ions in the *am-1a* strain is that ammonium ions were not accumulated by the mycelia as they were in the wild type strain. The uptake of ammonium ions by wild type and *am-1a* mycelia

Table 26

Nitrate Content of Extracts of Wild Type and *am-1a* Mycelia Which had been Exposed to Nitrate Medium Containing Different Concentrations of Ammonium Ions

Nitrogen source in induction medium	Nitrate content of extract			
	Wild type		*am-1a*	
	Total nmole/mycelial pad	Specific nmole/mg of protein	Total nmole/mycelial pad	Specific nmole/mg of protein
NO_3^- (10 mM)	75	2.0 (100)*	119	3.6 (100)
NO_3^- (10 mM) + NH_4^+ (5 mM)	81	1.7 (85)	94	2.2 (61)
NO_3^- (10 mM) + NH_4^+ (10 mM)	60	1.3 (63)	110	2.3 (64)
NO_3^- (10 mM) + NH_4^+ (20 mM)	119	2.7 (135)	81	2.0 (56)
NO_3^- (10 mM) + NH_4^+ (40 mM)	170	4.4 (220)	162	4.5 (125)

* Figures in parentheses are percentages of the nitrate content of extracts of mycelia induced in nitrate medium.

Mycelia pre-grown on casamino acids medium as described in Table 23, were induced for 3.5 hours in nitrate medium containing different concentrations of ammonium tartrate. The mycelia were harvested, extracted and the nitrate content of the extracts was determined as described in Methods. The results are representative of three experiments.

was examined by comparing the content of ammonia in extracts of wild type and *am-1a* mycelia exposed to nitrate medium containing different concentrations of ammonium ions. Wild type and *am-1a* mycelia exposed to nitrate media containing 5 mM, 10 mM or 20 mM ammonium ions for 3.5 hours contained similar concentrations of ammonia (Table 27). The concentration of ammonia in extracts of mycelia exposed to nitrate medium containing no ammonium ions was used as the base level for comparison. The presence of higher concentrations of ammonium ions (40 and 60 mM) in nitrate medium resulted in an increase in the concentration of ammonia in extracts of mycelia from both strains. The above results indicate that ammonium ions are taken up by *am-1a* mycelia and by wild type mycelia, and that they are capable of repressing nitrate reductase in both strains, although not to the same extent (Table 27).

2. Role of glutamic acid on the induction of nitrite reductase. The lack of repression of nitrite reductase by ammonium ions in strain *am-1a* may be due to a general inability to repress nitrite reductase by this strain. If so, metabolic products of ammonia, such as glutamic acid should be equally incapable of repressing nitrite reductase in strain *am-1a*.

The presence of glutamic acid in nitrate medium partially repressed the induction of nitrite reductase in wild type mycelia (Table 28). The extent of repression

Table 27

Concentration of Ammonia and Nitrate Reductase Activity in Extracts of Mycelia Induced on Basic Medium Containing 10 mM $NaNO_3$ Plus Different Concentrations of Ammonium Ions

Nitrogen sources in induction medium	Ammonia content of extract				NADPH-NR Specific activity (units/mg protein)	
	Wild type		*am-1a*			
	total*	specific†	total*	specific†	wild type	*am-1a*
NO_3^- (10 mM)	1230	23.6	1880	42.0	21.2	17.1
NO_3^- (10 mM) + NH_4^+ (5 mM)	1040	19.4	2120	32.6	6.75	9.8
NO_3^- (10 mM) + NH_4^+ (10 mM)	995	20.5	1785	41.0	6.12	8.0
NO_3^- (10 mM) + NH_4^+ (20 mM)	1175	20.8	2235	34.4	3.45	9.4
NO_3^- (10 mM) + NH_4^+ (40 mM)	2570	46.2	2480	46.4	2.0	9.6
NO_3^- (10 mM) + NH_4^+ (60 mM)	2745	48.1	2870	53.6	not done	

* nmole of nitrate per mycelial pad.
† nmole of nitrate per mg of protein.

Wild type and *am-1a* mycelial pads pregrown on CA medium were induced for 3.5 hours on nitrate medium containing 10 mM nitrite plus different concentrations of ammonium tartrate. The mycelia were harvested, extracted and assayed for NADPH-nitrate reductase activity. One ml aliquot of the extracts were used to determine the concentration of ammonia. Extracts from mycelia exposed to no nitrogen medium were used as blanks for the determination of ammonia. The NADPH-nitrate reductase activity shown is the average of duplicate measurements, and is representative of three experiments. The results for the concentrations of ammonia are also representative of three experiments.

Table 28

Effect of the Presence of Glutamic Acid in Nitrate Medium on the Induction of Nitrite Reductase in Wild Type and *am-1a* Mycelia

Nitrogen sources and concentrations in induction medium	Specific activity (units/mg protein)			
	Wild type		*am-1a*	
	nitrite reduction	ammonia production	nitrite reduction	ammonia production
NO_3^- (10 mM)	18.6	15.3	26.5	19.6
NO_3^- (10 mM) + glu (5 mM)	14.5	9.7	15.5	11.5
NO_3^- (10 mM) + glu (10 mM)	13.9	8.5	14.8	10.0
NO_3^- (10 mM) + glu (20 mM)	11.4	7.0	13.7	9.4
NO_3^- (10 mM) + glu (40 mM)	11.2	6.3	11.5	8.2

Pads of wild type and *am-1a* mycelia, pregrown in ammonia medium and CA medium respectively as described in Table 23, were induced for 9 hours on nitrate medium containing 10 mM nitrate plus different concentrations of glutamic acid, pre-neutralized with sodium bicarbonate. The mycelia were harvested, extracted and assayed as described in Methods. The results shown are representative of three experiments.

depended on the concentration of glutamic acid present in the induction medium. Nitrite reductase was similarly repressed by glutamic acid in *am-1a* mycelia. When glutamic acid was present in the induction medium at a concentration of 20 mM, the induction of nitrite reductase by nitrate was decreased by 50% in both wild type and *am-1a* mycelia.

When 1 mg of glutamic acid was present in the assay mixture, there was a 20% decrease in nitrite reductase activity observed with extracts of fully induced wild type mycelia (Table 29). It thus appears that some but not all of the repression of nitrite reductase by glutamic acid may be due to the inhibitory effect of the amino acid.

Therefore, nitrite reductase of *am-1a* strain is repressible. The result supports the suggestion that ammonium ions must be metabolized in order to repress nitrite reductase. Glutamate as a metabolic product of ammonia, is a plausible channel through which ammonium ions must pass in order to repress nitrite reductase.

3. Effect of other amino acids on the induction of nitrite reductase. Thirteen amino acids have been shown to repress the induction of nitrite reductase in *Lemna minor* (64). Hence, glutamic acid may not be the only metabolic product of ammonium ions that can repress nitrite reductase.

The effect of the presence of five different amino acids in nitrate medium on the induction of nitrite

Table 29

Effect of Amino Acids on Nitrite Reductase Activity of Extracts from Fully Induced Wild Type Mycelia

Additions to assay mixture	Specific activity (units/mg protein)	
	nitrite reduction	ammonia production
No addition	37.2	23.6
Alanine	36.7	27.7
Arginine	33.7	22.9
Glutamic acid	29.4	18.6
Glutamine	29.3	25.2
Glycine	33.9	23.5
Proline	33.9	16.9
Serine	35.0	17.1

Wild type mycelia, pregrown on ammonium medium as described in Table 23, were induced for 9 hours in nitrate medium containing 20 mM sodium nitrate. The mycelia were harvested, extracted and assayed for nitrite reductase activity in the absence and presence of different amino acids (1 mg/assay). The results shown are representative of three experiments.

reductase (AP activity) in wild type mycelia was examined. Arginine and glutamine at a concentration of 10 mM in nitrate induction medium displayed a 30% and a 55% repression of nitrite reductase, respectively (Table 30). There was an increase in the fully induced level of nitrite reductase activity in mycelia exposed to nitrate induction media containing alanine, glycine or proline.

When these amino acids were added singly to the nitrite reductase assay mixture at a concentration of 1 mg per 3 ml, only proline and serine caused inhibition of nitrite reductase activity by 30% (Table 29).

4. Summary. The above results showed that repression of nitrite reductase by ammonium ions is not due to inhibition of the enzyme activity. The lack of repression of nitrite reductase by ammonium ions in *am-1a* mycelia, the fact that nitrate and ammonia uptake are not affected in this strain, and the observation that glutamate represses nitrite reductase, all suggest that ammonium ions have to be metabolized in order to repress nitrite reductase. The effect of five amino acids on nitrite reductase was also studied. Only arginine and glutamine, when present in nitrate induction medium, repressed the formation of nitrite reductase in wild type mycelia. Proline, serine and glutamatic acid were the only amino acids examined which inhibited nitrite reductase activity *in vitro*.

Table 30

Effect of Five Different Amino Acids in Nitrate Induction Medium on the Induced Formation of Nitrite Reductase in Wild Type Mycelia

Nitrogen sources in induction medium	Specific activity (units/mg protein)	
	nitrite reduction	ammonia production
NO_3^-	14.2	10.1
NO_3^- + Alanine	13.0	10.5
NO_3^- + Arginine	6.7	4.8
NO_3^- + Glutamine	4.7	3.1
NO_3^- + Glycine	17.9	18.0
NO_3^- + Proline	22.6	20.4

Wild type mycelia pregrown on ammonia medium as described in Table 23, were induced for 9 hours in nitrate medium containing different amino acids (10 mM). The mycelia were harvested, extracted and assayed for nitrite reductase activity. The results shown are representative of three experiments.

DISCUSSION

A. The Nitrite Reductase Activity Which Does Not Produce Ammonia

1. The ND activity accounts for the lack of stoichiometry between nitrite reduction and ammonia production. The finding by Mulkins (40) that crude extracts of wild type mycelia exposed to nitrate medium catalyze the non-stoichiometric production of ammonia from nitrite was confirmed by this study. A nitrite reductase activity which does not produce ammonia (ND) was isolated from mycelial extracts by collodion membrane ultrafiltration, or by precipitation with 50% acetone. The ND activity is found in extracts of all strains of *Neurospora*, irrespective of their ability to utilize nitrite. In cell-free extracts of wild type mycelia exposed to nitrate medium, the ND activity could account for the difference between the nitrite reduced and the ammonia produced catalytically in the nitrite reductase assay (Table 4). An activity which catalyzes the reduction of nitrite to ammonia has also been identified. This activity was deduced to produce ammonia stoichiometrically from the reduction of nitrite, and is designated as AP (ammonia producing) activity. The AP

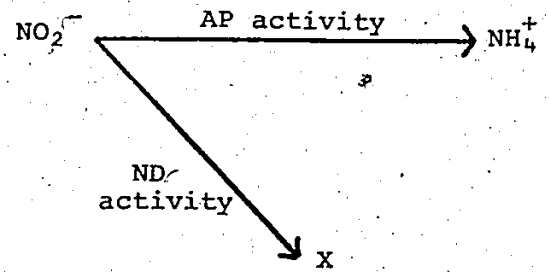
activity is found only in strains which can utilize nitrite, namely *nit-1*, *nit-3* and wild type. It thus appears that the AP activity is the physiologically important enzyme in the assimilation of nitrite.

The AP activity is present in extracts of strains which can utilize nitrite only when these are exposed to nitrite- or nitrate-containing media (Table 3). This suggests that the physiologically important nitrite reductase is inducible. Cook found earlier that the nitrite reducing activity was derepressible after a 17 hour incubation in the absence of nitrate (6). The higher specific activity observed in extracts of mycelia exposed to a medium containing no nitrogen source compared to that in extracts of mycelia exposed to a medium containing casamino acids may be due to the low protein content of extracts of mycelia that have undergone nitrogen starvation for a prolonged period of time. A similar observation was made by Garrett who noted that extracts of mycelia exposed for 17 hours to a nitrogen-deficient medium contained relatively low concentrations of protein (16). The specific activity of the ammonia producing enzyme reaches its peak between 8 and 12 hours of induction on nitrate or nitrite, and then starts to decline (Figures 6 and 7). Cook (6) did not find any ammonia produced from the reduction of nitrite catalyzed by extracts of mycelia that were derepressed for 17 hours, consequently,

most if not all of the nitrite reductase activity observed was probably due to the ND activity. This activity, as shown here, is present when *Neurospora* mycelia are exposed to any of the nitrogen sources.

In cases where nitrite non-utilizing strains (*nit-2*, *nit-4* ,*nit-5* and *nit-6*) were exposed to media containing any of the nitrogen sources used, or where nitrite utilizing strains (*nit-1*, *nit-3* and wild type) were exposed to media containing ammonia or no nitrogen source, the quantity of acetone non-precipitable ND activity observed was less than the total nitrite reducing activity found in the crude extracts (Table 14). There may be some acetone-precipitable nitrite reducing activity present in extracts of repressed, uninduced or mutant mycelia. Alternatively, the sensitivity of the ND activity to contact with acetone may be affected by the growth conditions of the mold. It should be noted here that the percentage recovery of the ND activity from crude extracts treated with acetone was estimated using the correction factor found with fully induced wild type mycelia, and that this may not be applicable to all situations.

The results just discussed confirm the proposal by Mulkins that there are at least two nitrite reductase activities in crude extracts of fully induced *Neurospora* mycelia: one which reduces nitrite to ammonia stoichiometrically (AP) and the other which reduces nitrite to something other than ammonia (ND).



More than one nitrite reductase activity has been reported in extracts of corn scutellum, of roots and of etiolated shoots of corn, of cultured tobacco cells and of Chlorella (9,25,29,75). These activities do not resemble the ND activity described in this work in molecular weight or in catalytic properties. An activity which reduces nitrite without producing ammonia has been observed to occur in extracts of citrus leaves, of excised tomato roots, and in other plant tissues (2,57,69). In all cases, it has been suggested that the activity was due to ascorbic acid. This is not likely in *Neurospora* because ND activity has different properties from ascorbic acid, as shown in the following section.

2. <u>The properties of ND activity</u>. The results obtained by treating the supernatant from acetone treated extracts with proteases suggest that the ND activity resides on a peptide. This interpretation requires some caution. The incubations were carried out at neutral pH, which is not optimal for the catalytic action of pepsin and of Sigma protease form VIII - the two proteases which caused inactivation of ND

activity. Pepsin has an optimal pH of about 3 (54). Sigma protease form VIII (also known as *Subtilisin carlsberg*) is an alkaline protease having a pH optimum of about 10 with casein as substrate (35). Thus the reduction in ND activity after incubation with these proteases may not have been due to the proteolysis. However, incubation of the ND activity with Sigma protease form VIII seems to have caused a reduction in its molecular size as revealed by a Sephadex G-25 chromatography (Figure 5). The loss of activity does not seem to be due to inactivation due to non-specific binding of the activity to proteins, since incubation of the acetone supernatant with bovine serum albumin resulted in no loss of activity.

It would seem reasonable from the foregoing to interpret the loss of ND activity during the incubation of the supernatant with pepsin and Sigma protease form VIII as a result of proteolysis; and that ND activity, consequently, resides on a peptide.

The presence of a sulfhydryl group(s) on the molecule responsible for ND activity was determined with two kinds of reagents. Iodoacetate, which causes alkylation of sulfhydryl groups, and p-chloromercurobenzoate, which forms a mercaptide with the sulfhydryl groups, both reduced the ND activity after 2 hours incubation in the presence of 10^{-2} hydrosulfite. The fact that iodoacetate has less of an effect on the ND activity in the absence of hydrosulfite than in its presence may suggest that the

ND activity resides on a molecule that has different conformations, a reduced one with the SH group exposed and an oxidized one where the SH group is unavailable for alkylation.

The presence of a metal in the molecule with ND activity is suggested by the effect of metal complexing agents on the ND activity. 2,2'-bipyridine and o-phenanthroline which can form complexes with metal-containing molecules, or which can remove metals from metal-containing molecules, inactivated the ND activity. Cyanide, which has a similar mode of action, also inactivated ND activity but at a higher concentration than 2,2'-bipyridine or o-phenanthroline. These agents have been widely used in demonstrating the presence of metals in enzymes (67). For instance, o-phenanthroline has been used to show that carboxypeptidase B is a metalloenzyme (15).

The nature of the ND activity demands further investigation. The present study suggests that it may be a small polypeptide (approximate M.W. 600) containing a sulfhydryl group and a metal. The metal may be iron, which under the conditions of the assay will catalyze nitrite reduction. We have found that 7.5 µmoles of hydrosulfite can reduce 1.5 µmoles of nitrite in twenty minutes in the presence of 0.4 µmoles of ferric chloride. In some ways the properties of the ND activity fit the description of siderochromes, which are hexapeptides

believed to be responsible for iron transport in *Neurospora* and other microorganisms (72). None of the known siderochromes, however, contain sulfhydryl groups (43).

B. Induction of Nitrite Reductase

Nitrate reductase has been shown to be induced by its substrate nitrite, and also by nitrate (Table 2). The role of nitrate in the induction of nitrite reductase has been found generally to be a direct one, except in *Anabaena* and radish cotyledons where nitrate has been reported to induce nitrite reductase indirectly.

1. Nitrate induces nitrite reductase directly. In *Neurospora*, Garrett (16) observed the simultaneous appearance of nitrate and nitrite reductases in nitrate induction medium, suggesting a direct role for nitrate in the induction of nitrite reductase. The observations in this study confirm Garrett's observation although a lag of one hour was observed before the appearance of nitrate and nitrite reductases. The presence of the lag may be due to the lower concentration of nitrate used in the induction medium in this study - 20 mM instead of the 80 mM employed in Garrett's experiments (16).

The direct role of nitrate as an inducer of nitrite reductase is further supported by the following observation.

The kinetics of induction of nitrite reductase in wild type mycelia in nitrate medium containing tungstate were similar to those observed during induction in the absence of tungstate, except that the peak activity was only half of that found in the normal case. In the presence of tungstate, the formation of active nitrate reductase was prevented. Therefore, there is a very small amount of NADPH-nitrate reductase activity formed - about 5% of that induced in medium containing nitrate alone. The maximum induced level of nitrite reductase formed by mycelia induced in nitrate medium containing tungstate, is higher than the corresponding level in mycelia induced in media containing nitrite at an optimal concentration. This would suggest that the appearance of nitrite reductase is probably not due to the production of nitrite from nitrate catalyzed by the very small amount of nitrate reductase formed in the presence of tungstate. The intracellular accumulation of nitrite in mycelia of *nit-6*, a strain which cannot utilize nitrite, induced in nitrate medium containing tungstate was lower than that of wild type mycelia exposed to 2 mM nitrite. Since a minimum of 2 mM nitrite was observed to be needed to induce nitrite reductase, it would seem that the intracellular amount of nitrite present in mycelia induced in nitrate medium plus tungstate is not sufficient to induce the observed formation of nitrite reductase.

Induction of nitrite reductase by nitrate is observed in mycelia of *nit-1* and *nit-3*, - both are strains which cannot utilize nitrate . . Strain *nit-1* has nitrate inducible NADPH-cytochrome c reductase activity and *nit-3* has BV-nitrate reductase activity, but neither of these strains possesses NADPH-nitrate reductase. Consequently, no nitrite can be produced by these strains. Hence, the appearance of nitrite reductase could be considered to be a direct effect of exposure of the mycelia to nitrate. The possible existence of trace, but undetectable low levels of nitrate reductase in these mutants should be entertained. The observation that the level of induced nitrite reductase is similar when the mycelia are induced on nitrite or on nitrate would suggest that a trace of nitrate reductase, if it exists, does not contribute much to the induction (Table 21).

The presence of ammonium ions in nitrate induction medium has been shown to repress the appearance of nitrite reductase in many systems (13,16,30,34,48,64,71). In *Neurospora*, ammonium ions in the culture medium repress the induction of nitrate and nitrite reductases, with the effect being greater on the induction of the former (Table 18). Based on the extrapolation of the experimental results, the induction of nitrate reductase is inhibited (repressed) completely by the presence of 70 mM ammonium ions in the nitrate induction medium. On the other hand, the induction

of nitrite reductase is reduced by only 50-60% under such conditions. Nitrite reductase is induced by nitrate in nitrite medium containing 100 mM ammonium ions, yet nitrate reductase formation is completely blocked under these conditions. Thus, no nitrite should be produced by the action of nitrate reductase (Table 18, part B).

2. Role of nitrate reductase in the induction of nitrite reductase. In a model postulated by Cove and Pateman (Figure 1), nitrate reductase was described as an effector which causes the conversion of an endogenously produced inducer to a repressor of both nitrate-and nitrite reductase. Conversion of the inducer to repressor is also caused by ammonium ions. When nitrate reductase is complexed with nitrate, it would block the above conversion. The model does not explain the observation that in the presence of ammonium ions, nitrite reductase is induced by nitrate in mutant strains which lack normal nitrate reductase.

Although active nitrate reductase is not made in the presence of tungstate in cultured tobacco cells, the induction of nitrite reductase by nitrate is normal, suggesting that normal nitrate reductase is not required for the induction of nitrite reductase (29). Nitrite reductase is induced by nitrate in *Neurospora* under the conditions that preclude the synthesis of active nitrate reductase (such as in *nit-1* and *nit-3* mycelia, or in wild type mycelia in the presence of tungstate, or of ammonium

ions). These observations suggest that active nitrate reductase is not required for the induction of nitrite reductase by nitrate. This is in agreement with the observations made in cultured tobacco cells but inconsistent with the reported model for *Aspergillus*. An involvement of active nitrate reductase in the induction of nitrite reductase by nitrate cannot be completely ruled out, because residual nitrate reductase activity which is present in tungstate-treated mycelia (5%) and in tungstate-treated cultured tobacco cells (10%), may be sufficient to cause induction of nitrite reductase. The relatively low specific activity of nitrite reductase observed when mycelia were induced in nitrate medium containing tungstate as compared to that induced in nitrate medium alone (1:2) could be explained if active nitrate reductase or some component thereof played some role in the induction of nitrite reductase.

Nitrite reductase is induced by nitrate in the presence of concentrations of ammonium ions that repress nitrate reductase completely. This observation suggests that even inactive nitrate reductase is not required for the induction of nitrite reductase by nitrate, however, the synthesis of all components of nitrate reductase may not be blocked by ammonium ions.

An alternative explanation for the presence of nitrite reductase in wild type mycelia induced in nitrate medium containing tungstate, or in *nit-1* and *nit-3* mycelia induced by nitrate is that some portion of the nitrate

reductase apoprotein plays a role in the induction of nitrite reductase. The observation that nitrite reductase is formed in *nit-1* and *nit-3* mycelia when they are exposed to media containing no nitrogen source seems to support this explanation. Both *nit-1* and *nit-3* have been shown to have partial nitrate reductase activities (NADPH-cytochrome c reductase and BV-nitrate reductase, respectively) whose formation does not require the presence of nitrate in the culture medium (61). These partial activities could be responsible for the "induced" formation of nitrite reductase in the absence of nitrate (or nitrite).

Strains *nit-1* and *nit-3* resemble some of the *cnx* and *nia D* strains of *Aspergillus* which can synthesize nitrite reductase in the absence of nitrate (47). However, the derepressed level of nitrite reductase in these two strains of *Neurospora* is much lower than the induced level of nitrite reductase in the wild type strain, suggesting that they are not quite analogous to the *cnx* or *nia D* strains of *Aspergillus*, which form the basis of Cove's model. Inactive nitrate reductase may function as an inefficient inducer of nitrite reductase in strains *nit-1* and *nit-3* incubated in the absence of ammonia.

In summary, nitrite reductase seems to be induced directly by nitrate in *Neurospora*. Normal nitrate reductase does not seem to be essential for the induction of nitrite reductase, as suggested by the model of Cove and Pateman, unless an undetectable level of the enzyme

is postulated to exist in the mycelia under all the conditions that were tested. The observations reported in this study are consistent with the following role of nitrate reductase in the induction of nitrite reductase: the enzyme enhances the induction of nitrite reductase but is not essential for it.

C. Repression of Nitrite Reductase

1. Ammonium ions repress the induction of nitrite reductase in wild type *Neurospora* mycelium. Ammonia has been shown to repress nitrite reductase in many systems (Table 2). Nitrite reductase (AP) activity is absent from extracts of wild type *Neurospora* mycelia exposed to ammonia. Ammonium ions when present in the nitrate medium partially repress nitrite reductase induction, the extent of repression depending on the concentration of ammonium ions present in the medium. The reasons for suggesting that this effect is due to repression rather than inactivation or inhibition of nitrite reductase are as follows: Mulkins has shown with desalted extracts of induced *Neurospora* mycelia that 3 mM ammonium chloride does not inhibit nitrite reductase activity (measured as nitrite reduction) *in vitro* (40). As AP activity makes up 80% of the total nitrite reduced, the observation shows that neither AP nor ND activity is inhibited by ammonium ions. Very high

ammonium ion concentrations (36 mM ammonium sulphate) in nitrite reductase assay mixture have been shown to cause a loss of 620 nmoles of nitrite in 25 minutes in the presence of reduced benzyl viologen (22). Such high concentration of ammonium ions is not comparable to the living system or to the assay described in this study.

The presence of ammonium ions in the culture medium did not stimulate *Neurospora* mycelia to produce extractable inhibitors of nitrite reductase activity. It would appear, consequently, that ammonium ions or a metabolic product of ammonium ions represses the accumulation of nitrite reductase in *Neurospora* mycelia. A similar conclusion has been drawn for the situation in *Chlorella* because nitrite reductase was not inactivated by ammonia *in vivo* (34).

2. Ammonium ions have no effect on the induction of nitrite reduction in *am-1a* mycelia. The presence of normally repressive concentrations of ammonium ions in the nitrate induction medium has virtually no effect on the formation of nitrite reductase in mycelia of strain *am-1a*, a glutamate dehydrogenaseless mutant which cannot assimilate ammonia (14). The lack of effect was apparently not due to any significant differences in the effect of ammonium ions on the intracellular concentration of nitrate or in the uptake of ammonium ions between *am-1a* and the wild type strain. The intracellular accumulation of nitrate

is inhibited slightly in both strains by the presence of low concentrations of ammonium ions in the culture medium (Table 26). At higher ammonium ion concentrations in the culture medium, there is an increase in the intracellular accumulation of nitrate in both strains, suggesting that an inhibition of nitrate uptake is not the cause of nitrite reductase repression by ammonium ions. The higher apparent intracellular accumulation of nitrate may be due to the repression of nitrate reductase by ammonium ions. Strain *am-1a* accumulated more ammonia in ammonia medium than the wild type strain. This is probably due to the absence in this strain of glutamate dehydrogenase which is the major channel for the assimilation of ammonia.

The observations on intracellular accumulation must be interpreted with caution. Contamination from cell wall-bound nitrate or ammonia cannot be eliminated at this stage. However, the appearance of nitrite in the induction media would suggest that nitrate is taken up and is reduced to nitrite by both strains. The fact that nitrate reductase is repressed by ammonium ions in both strains suggests that the intracellular ammonium ions are available for some type of metabolic activity in both strains.

3. Ammonium ions repress the induction of nitrite reductase indirectly. The simplest explanation for the lack of repression of nitrite reductase by ammonium ions in *am-1a* mycelia would be that unmetabolized ammonium ions

by themselves are not sufficient for repression. Two plausible alternatives can be entertained at this point.

1) Ammonium ions must first be metabolized by a pathway employing active glutamate dehydrogenase in order to repress nitrite reductase;
2) Ammonium ions plus some other component missing in strain *am-1a* are needed for repression of nitrite reductase.

Possibly 1) would seem attractive in view of the observation that glutamic acid, a metabolic product of ammonia, represses nitrite reductase in both *am-1a* and the wild type strain. The above observation also shows that nitrite reductase of strain *am-1a* is repressible. The fact that ammonium ions repress nitrate reductase in strain *am-1a* and the wild type strains also favours possibility 1).

C. The Effect of Other Amino Acids on the Induction of Nitrite Reductase

Five amino acids were tested, and of them, only arginine and glutamine repressed the induction of nitrite reductase by nitrate. These amino acids do not inhibit nitrite reductase activity *in vitro*. Proline, one of the three amino acids that showed significant inhibition of nitrite reductase activity *in vitro*, had no effect on the induction of nitrite reductase. The mechanism of repression by these amino acids is unknown at this time.

Heimer and Filner (19) have isolated a mutant line of tobacco cells (thr^-) in which the induction of nitrate reductase, but not the uptake of nitrate is inhibited by threonine. The amino acids examined in this study may act in the same way.

In summary, ammonium ions have been shown to repress the induction of nitrite reductase indirectly. Glutamic acid, a metabolic product of ammonia represses nitrite reductase in *am-1a* mycelia as well as in wild type mycelia. Arginine and glutamine when present in the nitrate induction medium also repress nitrite reductase. The mechanism of the repression is not known. It is believed that the repression of nitrite reductase is not due to an inhibition of nitrite reductase activity, or to an inhibition of nitrate uptake.

CONCLUSION

In conclusion, we would like to propose a model to describe the regulation of nitrite reductase in *Neurospora crassa* (Figure 12). According to the model, two nitrite reductase activities are present. ND activity reduces nitrite to something other than ammonia (X). This activity is present in all *Neurospora* strains under all conditions. AP activity reduces nitrite to ammonia stoichiometrically. This activity is directly induced by nitrite and by nitrate. The presence of nitrate reductase is not required for this induction. Nitrate reductase protein, on the other hand, can induce AP activity by itself. This is supported by the formation of AP activity in *nit-1* and *nit-3* mycelia when exposed to no nitrogen media. These strains are known to have derepressible partial nitrate reductase activities. Ammonium ions in our model do not have a direct effect on the formation of AP activity. The observed repression by ammonium ions is caused by their metabolic product(s). This is shown by the study with *am-1a* mycelia, a strain that cannot utilize ammonia. Three amino acids have been found to repress the formation of AP activity. However, there may be more than one type of repression involved.

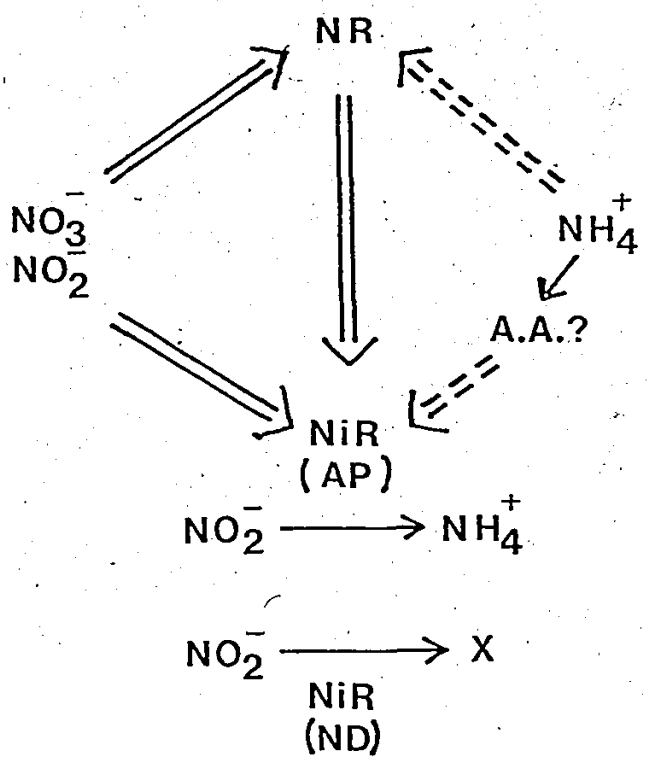


Figure 12. Model describing the regulation of nitrite reductase in *Neurospora crassa*.

Symbols:

⟹ induction.

⇢⇢⇢⇢⇢⟩ repression.

NR nitrate reductase.

NiR nitrite reductase.

A.A. amino acid.

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