Genetic and Biochemical Studies of Nitrate Reduction in *Aspergillus nidulans*

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1. In *Aspergillus nidulans* nitrate and nitrite induce nitrate reductase, nitrite reductase and hydroxylamine reductase, and ammonium represses the three enzymes. 2. Nitrate reductase can donate electrons to a wide variety of acceptors in addition to nitrate. These artificial acceptors include benzyl viologen, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride, cytochrome *c* and potassium ferricyanide. Similarly nitrite reductase and hydroxylamine reductase (which are possibly a single enzyme in *A. nidulans*) can donate electrons to these same artificial acceptors in addition to the substrates nitrite and hydroxylamine. 3. Nitrate reductase can accept electrons from reduced benzyl viologen in place of the natural donor NADPH. The NADPH-nitrate-reductase activity is about twice that of reduced benzyl viologen-nitrate reductase under comparable conditions. 4. Mutants at six gene loci are known that cannot utilize nitrate and lack nitrate-reductase activity. Most mutants in these loci are constitutive for nitrite reductase, hydroxylamine reductase and all the nitrate-induced NADPH-diaphorase activities. It is argued that mutants that lack nitrate-reductase activity are constitutive for the enzymes of the nitrate-reduction pathway because the functional nitrate-reductase molecule is a component of the regulatory system of the pathway. 5. Mutants are known at two gene loci, *niiA* and *niiB*, that cannot utilize nitrite and lack nitrite-reductase and hydroxylamine-reductase activities. 6. Mutants at the *niiA* locus possess inducible nitrate reductase and lack nitrite-reductase and hydroxylamine-reductase activities. It is suggested that a single enzyme protein is responsible for the reduction of nitrite to ammonium in *A. nidulans* and that the *niiA* locus is the structural gene for this enzyme. 7. Mutants at the *niiB* locus lack nitrate-reductase, nitrite-reductase and hydroxylamine-reductase activities. It is argued that the *niiB* gene is a regulator gene whose product is necessary for the induction of the nitrate-utilization pathway. The *niiB* mutants either lack or produce an incorrect product and consequently cannot be induced. 8. Mutants at the *niiR* locus cannot utilize nitrate or nitrite unless provided with a flavine supplement. When grown in the absence of a flavine supplement the activities of some of the nitrate-induced enzymes are subnormal. 9. The growth and enzyme characteristics of a total of 123 mutants involving nine different genes indicate that nitrate is reduced to ammonium. Only two possible structural genes for enzymes concerned with nitrate utilization are known. This suggests that only two enzymes, one for the reduction of nitrate to nitrite, the other for the reduction of nitrite to ammonium, are involved in this pathway.

The utilization of nitrate is an important metabolic process, since nitrate is the chief source of nitrogen for higher plants and is readily metabolized by many micro-organisms. It is usually assumed that nitrate is reduced to ammonium as follows:

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The nature of the postulated intermediate between nitrite and hydroxylamine is uncertain (Nicholas, 1963). One possibility is hyponitrite, which has the appropriate oxidation state of +1 for nitrogen, and there is a report of hyponitrite reductase in *Neurospora* (Medina & Nicholas, 1957). Although the pathway outlined seems the most likely, there are two further possibilities that at present cannot be ruled out. It may be that, *in vivo*, one or more intermediates are not reduced in the free form, but are bound to some organic compound

or are never released in the free form from an enzyme aggregate. These ideas are attractive since the postulated intermediates between nitrite and hydroxylamine are unstable or toxic or both. The other possibility is that nitrate is not reduced completely to ammonium, but that some intermediate after nitrite is used for amino acid synthesis. It has, for example, been suggested that hydroxylamine could be used to form oximes, which could then be reduced to amino acids (Saris & Virtanen, 1957*a,b*). One of the ways in which more definitive information may be obtained about the utilization of nitrate is by the use of biochemical mutants, and this is one of the objectives of the present work.

The first step in the utilization of nitrate is the reduction of nitrite by the enzyme nitrate reductase [NAD(P)H-nitrate oxidoreductase, EC 1.6.6.3]. This enzyme has been found in higher plants and many micro-organisms. It is probable that the enzyme catalyses the transfer of electrons from reduced nicotinamide nucleotides via flavine, iron and molybdenum to nitrate (Nicholas, 1963):

In *Neurospora* Kinsky & McElroy (1958) have shown that a 70-fold purification of nitrate reductase did not separate nitrate-reductase activity from cytochrome *c*-reductase activity (NADPH-cytochrome *c* oxidoreductase, EC 1.6.2.3) and suggested that a single enzyme protein was involved. In *Aspergillus nidulans* it has been shown that nitrate-reductase and cytochrome *c*-reductase activities are only present at a high level in wild-type mycelium that has been grown in the presence of nitrate and absence of ammonia (Pateman, Cove, Rever & Roberts, 1964; Cove, 1966). It is reasonable to suppose that nitrate reductase can donate electrons from some intermediate point on the transport chain, probably the flavine, to cytochrome *c* and thus exhibit cytochrome *c*-reductase activity. It has also been shown in *A. nidulans* (Pateman *et al.* 1964) that a total of five genetically different types of mutants lacked normal nitrate-reductase activity as a result of a cofactor deficiency. All of these types of mutants possessed high activities of cytochrome *c* reductase, i.e. were constitutive, when grown in the absence of nitrate. It was argued that these mutants, which lacked nitrate-reductase activity, were constitutive for the synthesis of nitrate-reductase protein and that this resulted in the observed high cytochrome *c* reductase activity after growth in the absence of nitrate. It seemed that this system was a favourable one for investigating the regulation of enzyme synthesis in a fungus and work was continued with that object in view. This paper presents data on

a number of NADPH-diaphorase activities, NADPH-nitrate oxidoreductase (EC 1.6.6.3), NADPH-nitrite oxidoreductase (EC 1.6.6.4), NADPH-hydroxylamine oxidoreductase (EC 1.6.6.4) and benzyl viologen-nitrate oxidoreductase in *Aspergillus* mycelium grown in the presence and absence of nitrate. These enzyme activities were determined in a strain prototrophic with respect to nitrate utilization and in 11 different types of mutant all of which were abnormal in some way with respect to the utilization of nitrate.

MATERIALS AND METHODS

Aspergillus strains

A translocation-free biotin-requiring strain of *A. nidulans*, *bi-1* obtained from the Department of Genetics, University of Glasgow, was used as the prototrophic strain with respect to nitrogen metabolism. A total of nine genetically different strains of *A. nidulans* that can use nitrite, but not nitrate, as a sole source of nitrogen were used. The mutant *niaD-17* lacked nitrate-reductase activity and belonged to the *D* group of non-nitrate-utilizing mutants (Pateman *et al.* 1964). The *niaD* locus is probably the structural gene for nitrate reductase in *Aspergillus*. The mutants *cnx A*, *cnx B*, *cnx C*, *cnx E*, *cnx F*, *cnx G* and *cnx H* lacked both nitrate-reductase and xanthine-dehydrogenase activities and belong respectively to the *A-H* groups of mutants reported previously (Pateman *et al.* 1964). It is considered that all of these types of mutants are abnormal with respect to a common factor that is essential for both nitrate-reductase and xanthine-dehydrogenase activity, and they have been designated *cnx* mutants. It has been shown that mutation in at least five gene loci can result in the *cnx* phenotype (Rever, 1965; Pateman *et al.* 1964); none of these loci represents the structural gene for either nitrate reductase or xanthine dehydrogenase. Three genetically different strains of *A. nidulans*, *nii A-2*, *nii B-1* and *nii ribo A-1* were used, each of which can use ammonium but not nitrate nor nitrite. The *cnx* loci are distributed over five of the eight chromosomes of *A. nidulans*. The *niaD*, *nii A* and *nii B* loci are all on chromosome VIII with *niaD* and *nii A* probably about 10 cM apart and separated from *nii B* by about 40 cM. The *nii ribo A* locus is also on chromosome VIII (Rever, 1965). Each type of mutant complements in heterocaryons with all of the others and consequently is recessive in function (Cove & Pateman, 1963; Rever, 1965).

Culture of mycelium and preparation of cell-free extracts

Mycelium was grown in shaken culture at 25° and harvested, and cell-free extracts were prepared as described by Cove (1966).

Chemicals

Wherever possible analytical-grade chemicals were used.

Enzyme assays

The optimum substrate concentrations and the composition of buffer for all the following assays were determined by

experiments with cell-free extracts of the prototrophic strain. In all cases the rate of reaction was strictly proportional to the concentration of cell-free extract under the conditions stated.

NADPH-nitrate oxidoreductase. The method used was basically that described by Nason & Evans (1953) in which the nitrate produced during the reaction at 25° is estimated colorimetrically. The detailed procedure is described by Cove (1966).

NADPH-nitrite oxidoreductase. The oxidation of NADPH was measured by the change in E_{340} in a recording spectrophotometer. The assay mixture was: 0.2 mg. of NaNO_2 ; 0.3 mg. of NADPH; 35 μg . of FAD; 50–100 μl . of cell-free extract; 0.1 M-sodium orthophosphate buffer, pH 7.0, to a final volume of 1.5 ml. The initial rate of reaction was determined in the presence and absence of nitrite at 25°.

NADPH-hydroxylamine oxidoreductase. The oxidation of NADPH was measured by the change in E_{340} . The assay mixture was: 7.0 mg. of hydroxylamine hydrochloride; 35 μg . of FAD; 0.3 mg. of NADPH; 50–100 μl . of cell-free extract; 0.01 M-sodium pyrophosphate buffer, pH 8.5, to a final volume of 1.5 ml. The initial rate of reaction was determined in the presence and absence of hydroxylamine at 25°.

NADPH-cytochrome c oxidoreductase. The reduction of cytochrome c was measured by the change in E_{551} . The assay mixture was that given by Cove & Coddington (1965) except that 35 μg . of FAD was used. The rate of reaction during the first 30 sec. at 25° was used as the measure of enzyme activity.

NADPH-benzyl viologen oxidoreductase. The oxidation of NADPH was measured by the change in E_{340} . The assay mixture was: 4.0 mg. of benzyl viologen; 0.6 mg. of NADPH; 35 μg . of FAD; 10–100 μl . of cell-free extract; 0.1 M-sodium orthophosphate buffer, pH 8.0, to a final volume of 1.5 ml. The rate of reaction during the first 30 sec. at 25° was used as the measure of enzyme activity.

NADPH-2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-tetrazolium chloride oxidoreductase. The reduction of the tetrazolium was measured by the change in E_{500} . The assay mixture was: 2 mg. of the tetrazolium; 1.0 mg. of NADPH; 35 μg . of FAD; 10–100 μl . of cell-free extract; 0.1 M-sodium orthophosphate buffer, pH 8.0, to a final volume of 1.5 ml. The linear rate of reaction during the first 60 sec. at 25° was used as the measure of enzyme activity.

NADPH-potassium ferricyanide oxidoreductase. The reduction of the ferricyanide was measured by the change in E_{420} . The assay mixture was: 1 mg. of $\text{K}_3\text{Fe}(\text{CN})_6$; 1 mg. of NADPH; 35 μg . of FAD; 10–100 μl . of cell-free extract; 0.1 M-sodium orthophosphate buffer, pH 8.0, to a final volume of 1.5 ml. The rate of reaction during the first 30 sec. at 25° was used as the measure of enzyme activity.

Benzyl viologen-nitrate oxidoreductase. The method was similar to that of Lowe & Evans (1964) and the nitrite produced during the reaction at 25° estimated colorimetrically as for NADPH-nitrate reductase. The assay mixture was: 20 mg. of NaNO_3 ; 3.5 μg . of FAD; 0.4 mg. of benzyl viologen; 3 mg. of $\text{Na}_2\text{S}_2\text{O}_4$; 50–200 μl . of cell-free extract; 0.05 M-sodium orthophosphate buffer, pH 7.5, to a final volume of 2.5 ml. The reaction was started by adding cell-free extract immediately after the dithionite followed by gentle mixing in uncapped tubes (8 cm. $\times$ 1 cm.). The tubes were kept in a 25° water bath for 5 min., the dithionite being

sufficient to keep the benzyl viologen fully reduced for this period. The reaction was stopped by vigorous mixing, which results in complete oxidation of the benzyl viologen in 10–30 sec. The nitrite in the assay mixture was compared with a blank tube from which the benzyl viologen was omitted.

Enzyme units

All units are expressed as $\text{m}\mu\text{moles}$ of substrate transformed/min. Specific activities are in enzyme units/mg. of protein.

Protein determination

The amount of soluble protein in cell-free extracts was estimated by the modification of the Folin method described by Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Growth responses

All the strains used were tested for growth with various compounds as sole nitrogen sources by inoculation of conidia on to solid agar medium. The growth responses were scored after inoculation for 48 hr. at 37°. The results are given in Table 1.

Enzyme activities

For enzyme assays all the strains were grown in shaken culture at 25° on liquid medium. Unless otherwise stated the medium contained 5 mM-urea as the only initial source of nitrogen. Mycelium described as grown on urea had no further nitrogen added during growth; additional nitrogen sources were added 15 hr. after inoculation at a concentration of 10 mM for sodium nitrate or ammonium

Table 1. *Growth of the prototroph and mutant strains on various nitrogen sources*

Growth was scored as normal (+) or very slight (–) on solid agar medium after incubation for 48 hr. at 37°.

Genotype	NO_3^-	NO_2^-	NH_4^+	Hypo-xanthine	Uric acid
Prototroph	+	+	+	+	+
<i>cnx A-5</i>	–	+	+	–	+
<i>cnx B-11</i>	–	+	+	–	+
<i>cnx C-2</i>	–	+	+	–	+
<i>cnx E-4</i>	–	+	+	–	+
<i>cnx F-8</i>	–	+	+	–	+
<i>cnx G-4</i>	–	+	+	–	+
<i>nia D-17</i>	–	+	+	+	+
<i>nii A-1</i>	–	–	+	+	+
<i>nii B-1</i>	–	–	+	+	+
<i>nii ribo A-1</i>	–	–	+	+	+
<i>nii ribo A-1*</i>	+	+	+	+	+

* Medium supplemented with 2.5 μg . of riboflavine/ml.

Table 2. *Enzyme activities of the prototroph and mutant strains grown on various nitrogen sources*

Cell-free extracts were prepared from mycelium grown as follows: (1) 5 mm-urea as initial and only nitrogen source, mycelium harvested at 20 hr., (2) 5 mm-urea as initial nitrogen source, 10 mm-NaNO₃ added at 15 hr., mycelium harvested at 20 hr.; (3) 5 mm-urea as initial nitrogen source, 10 mm-NH₄NO₃ added at 15 hr., mycelium harvested at 20 hr. The activities of nitrate reductase (NO₃R), nitrite reductase (NO₂R), hydroxylamine reductase (NH₂OH R), cytochrome c reductase (CR), benzyl viologen reductase (BV R), iodophenylnitrophenyltetrazolium reductase (INT R) and potassium ferriyanide reductase (FCNR) were determined and are given in μ moles of substrate transformed/min./mg. of protein.

	Enzyme activities											
	(1) Mycelium grown on urea						(2) Mycelium grown on urea + NaNO ₃					
	NO ₃ R	NO ₂ R	NH ₄ OH R	BV R	CR R	INT R	FCNR R	NO ₃ R	NO ₂ R	NH ₄ OH R	BV R	CR R
Prototroph	4	2	3	66	268	151	383	75	136	146	524	528
Prototroph	4	1	15	30	169	162	466	91	151	224	550	907
<i>crzA-5</i>	0	199	319	627	508	710	2631	0	261	330	667	327
<i>crzB-11</i>	0	225	345	655	380	906	3200	0	239	258	659	352
<i>crzC-2</i>	0	100	120	280	480	798	3530	0	152	143	381	741
<i>crzE-2</i>	<1	10	22	80	209	—	—	4	269	340	1400	1180
<i>crzE-3</i>	0	153	161	1110	828	—	—	0	131	209	1260	971
<i>crzE-4</i>	<1	268	352	1800	1890	—	—	<1	272	340	1630	1970
<i>crzE-13</i>	0	238	281	1260	1210	—	—	0	257	307	1540	1360
<i>crzE-14</i>	0	4	26	210	248	265	1313	0	276	381	1605	1960
<i>crzF-1</i>	0	108	157	668	565	—	—	0	156	227	792	737
<i>crzF-2</i>	0	224	253	924	985	—	—	0	195	219	1063	897
<i>crzF-8</i>	0	108	205	813	716	833	3800	0	148	223	840	475
<i>crzG-2</i>	2	<1	15	22	153	—	—	2	212	254	643	975
<i>crzG-4</i>	0	174	252	816	1110	543	2605	0	184	247	894	1060
<i>crzH-3</i>	<1	192	284	1195	1290	706	3050	<1	143	167	794	780
<i>niaD-17</i>	0	137	246	1030	470	1204	4450	0	102	139	530	537
<i>niaA-1</i>	1	3	9	114	89	114	424	76	2	21	309	327
<i>niaB-2</i>	2	3	24	36	82	146	397	2	2	21	44	90
<i>nitroA-1</i>	3	0	13	74	209	—	—	72	33	92	320	805
<i>nitroA-1*</i>	2	1	7	103	87	—	—	113	217	314	577	506

* All media supplemented with 2.5 μ g. of riboflavin/ml.

nitrate and 5mM for ammonium tartrate. All mycelia were harvested 20, 21 or 23hr. after inoculation. The enzyme activities in mycelia grown on various media are given in Tables 2, 3 and 4.

Prototroph. Nitrate-reductase activity was considerably higher in mycelium grown in the presence of nitrate than in mycelium grown on urea alone.

Table 3. Induction of nitrate reductase, nitrite reductase and hydroxylamine reductase by low concentrations of nitrate and nitrite

Mycelium of the strain *bi-1* was grown on medium containing 5mM-urea for 20hr. at 37°; the inducer was then added at the appropriate concentration, and 1hr. after the addition of the inducer the mycelium was harvested. The enzyme activities in the mycelium from the various treatments are recorded in μ moles of substrate transformed/min./mg. of protein.

Inducer	Enzyme activities		
	Nitrate reductase	Nitrite reductase	Hydroxylamine reductase
None	4	0	6
100 μ M- NO_3^-	52	22	40
10 μ M- NO_3^-	51	27	56
100 μ M- NO_2^-	32	17	14
10 μ M- NO_2^-	22	22	31

These results demonstrate that nitrate reductase is induced by nitrate in *Aspergillus*, as shown previously (Pateman *et al.* 1964; Cove, 1966). The nitrate-reductase activity on mycelium grown on ammonium nitrate is lower than in mycelium grown on nitrate; this is in agreement with the finding (Cove, 1966) that nitrate reductase in *Aspergillus* is repressed by ammonia. Table 3 shows that both nitrate-reductase and nitrite-reductase activities are higher in mycelium grown in the presence of nitrite than in its absence. This demonstrates that nitrite induces both nitrate reductase and nitrite reductase. It is not known if nitrite can induce hydroxylamine reductase. In the presence of nitrite it is possible that hydroxylamine will be produced and this might be responsible for the observed induction of hydroxylamine reductase. It is not known if hydroxylamine is an inducer of nitrate reductase, nitrite reductase or hydroxylamine reductase. Hydroxylamine is extremely toxic for *A. nidulans* and in the very low concentrations that can be tested does not appear to be an available source of nitrogen.

All of the NADPH-diaphorase activities were quite high in mycelium grown on urea alone although the activities were far higher in the presence of nitrate. The 'diaphorase' activities are non-specific and the uninduced activities in mycelium grown on urea were probably largely composed of diaphorase activities exhibited by

Table 4. NADPH-nitrate reductase and reduced benzyl viologen-nitrate reductase in the prototroph and various mutants

Cell-free extracts were prepared from mycelium grown as follows: (1) 5mM-urea as initial and only nitrogen source, mycelium harvested at 20hr.; (2) 5mM-urea as initial nitrogen source, 10mM- NaNO_3 added at 15hr., mycelium harvested at 20hr. The activities of NADPH-nitrate reductase (NADPH- NO_3R) and reduced benzyl viologen-nitrate reductase (BVH- NO_3R) were determined and are given in μ moles of substrate transformed/min./mg. of protein. The enzyme activities of 12 independently mutated *nitB* mutants, *nitB-1* to *nitB-12*, are given together since they are all within a small range.

Genotype	Enzyme activities			
	(1) Mycelium grown on urea		(2) Mycelium grown on urea + NaNO_3	
	NADPH- NO_3R	BVH- NO_3R	NADPH- NO_3R	BVH- NO_3R
Prototroph	1.3	1.8	63	37
<i>cnx A-5</i>	0	1.6	0	1.4
<i>cnx B-11</i>	0	2.1	0	2.0
<i>cnx C-2</i>	0	1.0	0	1.4
<i>cnx E-14</i>	0	1.4	0	0.9
<i>cnx F-2</i>	0	0.9	0	1.0
<i>cnx G-2</i>	1.9	1.0	1.7	1.4
<i>cnx H-3</i>	0.4	1.0	0.5	0.9
<i>nia D-17</i>	0	1.1	0	1.3
<i>nit A-1</i>	1.1	2.9	76	28
<i>nitB-1</i> to <i>nitB-12</i> inclusive	1.0-3.0	1.0-3.0	1.0-3.0	1.0-3.0
<i>nitB0 A-1</i>	1.9	1.5	60	26

enzymes not connected with the utilization of nitrate.

Mutants niaD and cnx. These strains grew on nitrite but not on nitrate as sole source of nitrogen. They all lacked nitrate-reductase activity when grown on urea or on urea plus nitrate. Most of these strains when grown either on urea or on urea plus nitrate possessed enzyme activities, except for nitrate reductase, comparable with those of the prototroph grown on urea plus nitrate. Thus these strains, which lacked normal nitrate-reductase activity, were constitutive for the rest of the enzymes normally induced by nitrate. The strain *cnxE-14* is an example of the minority of strains in which all the enzymes except nitrate reductase were still inducible by nitrate in a similar fashion to the prototrophic strain. The characteristics of these 'inducible' *cnx* strains demonstrate that nitrate is an inducer of nitrite reductase and hydroxylamine reductase since the mutants cannot reduce nitrate.

Table 2 shows that the *cnxE* mutants possessed very high levels of all the NADPH-diaphorase activities. This is characteristic of all 14 known members of this group. The *cnxE* mutants can be repaired by high concentrations of molybdenum in the medium and can then utilize nitrate as a sole nitrogen source (Cove, Pateman & Rever, 1964). The other types of *cnx* mutants are not repaired by molybdenum. It may be that the special defect of the *cnxE* mutants with respect to the molybdenum cofactor results in an enhanced ability to donate electrons to other electron acceptors.

Mutant niiA. This strain did not grow on nitrate or nitrite as sole source of nitrogen. It possessed similar nitrate-inducible nitrate reductase to the prototrophic strain. The nitrite-reductase and hydroxylamine-reductase activities are very low in mycelium grown either on urea or urea plus nitrate. It is clear that the inability of strain *niiA* to produce functional nitrite reductase or hydroxylamine reductase is responsible for the failure to grow on nitrate or nitrite.

Mutant niiB. This strain is similar to strain *niiA*, in that it grew on ammonium or urea, but not on nitrate or nitrite. It produced readily detectable but low levels of all the enzyme activities when grown on urea or urea plus nitrate. The low enzyme activities account for the inability of strain *niiB* to grow on nitrate or nitrite.

Mutant niiriboA. This mutant grew almost as well as the prototroph on nitrogen sources such as urea or ammonium. It did not grow on nitrate or nitrite unless the medium was supplemented with flavine, riboflavine being the most effective form used. The nitrite-reductase and hydroxylamine-reductase activities of strain *niiriboA* were sub-normal when grown on urea plus nitrate compared with those of the prototroph on the same medium.

The enzyme activities of strain *niiriboA* grown on urea plus nitrate with a flavine supplement were fully comparable with those of the prototroph.

Effect of ammonium on enzyme activities

It has been shown previously that nitrate reductase in prototrophic strains of *A. nidulans* is induced by nitrate and repressed by ammonium (Cove & Pateman, 1963; Cove, 1966). The prototrophic strain grown on ammonium, or urea plus ammonium, possesses activities of nitrate reductase and all the other enzymes lower than those shown on urea alone. The enzyme activities of the prototroph grown on urea plus ammonium nitrate are considerably lower than when growth is on urea plus nitrate (Table 2). Ammonium greatly decreases the enzyme activities although it does not completely override the inducing effect of nitrate. The enzyme activities of the 'constitutive' *niaD* and *cnx* mutants are considerably lower when grown on urea plus ammonium nitrate than on urea or urea plus nitrate. This demonstrates that the constitutive synthesis of these enzymes is repressed to a considerable extent by ammonium in these mutants.

Benzyl viologen-nitrate-reductase activities

The prototrophic strain, a total of 12 *niiB* mutants and one representative of each of the other types of mutants were assayed for this enzyme activity (Table 4). The prototroph and strains *niiA* and *niiriboA* possessed benzyl viologen-nitrate-reductase activity when grown on urea plus nitrate. The specific activity of the benzyl viologen-nitrate reductase in each case was about half that of the corresponding NADPH-nitrate reductase. The chief objective of this analysis was to discover if any group of mutants (and in particular the *niiB* group) possessed benzyl viologen-nitrate-reductase but not NADPH-nitrate-reductase activity. Table 4 shows that in all cases either both activities were present or both activities were very low.

DISCUSSION

The four NADPH-diaphorase activities investigated were all increased threefold or more by the presence of nitrate during growth and this induction by nitrate was partially overcome by ammonium. These enzyme activities therefore are induced by nitrate and repressed or inhibited (or both) by ammonium in a similar fashion to nitrate reductase, nitrite reductase and hydroxylamine reductase. This is strong evidence that the enzyme or enzymes that exhibit these diaphorase activities play some role in the utilization of nitrate *in vivo*. It has been shown in *Neurospora* (Kinsky & McElroy, 1958)

that material purified 70-fold with respect to nitrate-reductase activity retains NADPH-cytochrome *c*-reductase activity. In *A. nidulans* Cove & Coddington (1965) showed that nitrate reductase purified 300-fold possesses cytochrome *c*-reductase activity and that the ratio of the two activities was constant during the later stages of the purification. This demonstrated that nitrate reductase is responsible for at least part of the nitrate-induced cytochrome *c*-reductase activity in *A. nidulans*. Preliminary experiments have shown that purified nitrate reductase from *A. nidulans* prepared by the method of Cove & Coddington (1965) possessed high levels of the other NADPH-diaphorase activities in addition to cytochrome *c*-reductase activity. It is reasonable then to suppose that nitrate reductase can mediate the transfer of electrons to other acceptors in addition to cytochrome *c* and is responsible for part at least of the other NADPH-diaphorase activities. It is also probable that nitrite reductase is responsible for some part of the nitrate-induced diaphorase activities. This enzyme is similar to nitrate reductase in that electrons from the donor NADPH are probably passed along a transport chain to the substrate. It is likely that various electron acceptors can take electrons from one or more intermediate points on this chain in a similar fashion to that postulated for nitrate reductase. During the initial stages of purification of *Aspergillus* nitrate reductase by ammonium sulphate precipitation the protein fraction precipitated between 30% and 45% saturation contains high nitrite-reductase, cytochrome *c*-reductase and other NADPH-diaphorase activities, but little nitrate reductase (D. J. Cove, & J. A. Pateman, unpublished work). The enzyme characteristics of the *niiA* and *niiB* mutants strongly support the hypothesis that both nitrate reductase and nitrite reductase can exhibit a variety of NADPH-diaphorase activities. When the prototroph and an *niiA* strain are grown in the presence of nitrate, the latter strain lacks nitrite reductase and hydroxylamine reductase and exhibits about 50% of the diaphorase activities of the prototroph. It is probable that this fall in nitrate-induced diaphorase activity is due to the absence of nitrate-induced nitrite reductase and hydroxylamine reductase from the *niiA* mutant. An *niiB* mutant grown in the presence of nitrate lacks nitrate reductase, nitrite reductase and hydroxylamine reductase, and all the diaphorase activities are considerably less than those of an *niiA* mutant grown on nitrate. It is probable that the difference in nitrate-induced diaphorase activity between the *niiA* and *niiB* mutants is due to the presence in the former and absence from the latter of nitrate reductase. Thus the available evidence indicates that nitrate reductase, nitrite reductase

and possibly hydroxylamine reductase can donate electrons to a variety of acceptors in addition to their primary substrates. It seems that nitrate reductase is responsible for about 50% of the observed nitrate-induced diaphorase activity, with nitrite reductase and hydroxylamine reductase being responsible for the rest. A remarkable feature of this situation is the lack of specificity of the *Aspergillus* nitrate reductase and nitrite reductase with respect to the artificial electron acceptors. These range from potassium ferricyanide with $E'_0 + 0.48$ v to benzyl viologen with $E'_0 - 0.314$ v, the latter being close to the potential $E'_0 - 0.32$ v of NADPH, the natural electron donor. The flavine moiety is the most likely point on the electron-transport chain of both enzymes from which electrons could be donated to the artificial acceptors. All the diaphorase activities are markedly dependent on the presence of FAD in the assay system for maximum activity. This FAD that participates in the diaphorase reactions must be bound in some fashion to the enzymes. Reduced FAD in the free state is rapidly autoxidizable. Therefore if the enzymic reactions resulted in free reduced FAD this would be oxidized by molecular oxygen and result in an NADPH-FAD-oxido-reductase activity. *Aspergillus* cell-free extracts do not appreciably oxidize NADPH in the presence of FAD and the absence of any other electron acceptor.

It has been shown that enzyme activities that are induced by nitrate in the prototrophic strain are constitutive in a variety of genetically different mutants. It is probable that the activities of all these enzymes in the cell are determined by a common control system, some element of which is not functioning correctly in the mutants. The only characteristic shared by all the groups of mutants is a lack of nitrate-reductase activity; it must be assumed that this is responsible for the constitutive synthesis of nitrite reductase, hydroxylamine reductase and probably an altered nitrate reductase. There are two hypotheses that could account for this situation.

(1) It has been shown that nitrate can induce both nitrate reductase and nitrite reductase. Thus the synthesis of all three enzyme proteins in the mutants might be due to the accumulation of a low concentration of nitrate in cells that lack functional nitrate-reductase activity. The presence of nitrate in cells grown in the absence of nitrate could be due to the production of small amounts of nitrate from ammonium or the concentration of trace amounts of nitrate (or both) that might be present in various constituents of the growth medium. There are two lines of evidence that are not easily reconcilable with this hypothesis. First, it has been shown that at low concentrations nitrite is an inducer comparable with nitrate (Table 3). Consequently, if *niiA* and

cnx mutants are constitutive because of a constant low concentration of nitrate, then *niiA* mutants should be constitutive for nitrate reductase because of a constant low concentration of nitrite, since *niiA* mutants lack nitrite reductase. However, *niiA* mutants are inducible for nitrate reductase (Table 2). Secondly, it has been shown that the *niiA* and *cnx* mutants are more sensitive to repression by ammonium than the prototrophic strain (Cove, 1967); i.e. mutants at different gene loci, whose only common characteristic is lack of nitrate-reductase activity, are altered with respect to ammonium repression. This finding strongly implies that nitrate reductase has some control function (see hypothesis 2 below).

(2) It may be that nitrate reductase, in addition to its catalytic function, is a component of a regulatory system that determines the rate of synthesis of all the nitrate-induced enzymes (Cove *et al.* 1964; Pateman, Rever & Cove, 1965; Cove, 1967).

Although most of the mutants that lack nitrate reductase are constitutive for the other enzymes, there are exceptions in all the groups of mutants. The existence of 'inducible' mutants that lack nitrate reductase and are inducible for the other enzymes is readily accounted for by either of the two hypotheses proposed. If the first hypothesis is correct, the inducible mutants are the result of mutations that do not completely abolish nitrate-reductase activity and consequently the residual enzyme activity removes the endogenous nitrate. If the second hypothesis is correct, the inducible mutants would be the result of mutations that almost abolish the enzymic activity of the nitrate-reductase molecule, but leave the regulatory activity unimpaired.

It is clear from the correlation between the growth and enzyme characteristics of the various mutants that the enzymes nitrate reductase, nitrite reductase and hydroxylamine reductase are essential for utilization of nitrate by *Aspergillus*. The mutants do not establish that nitrite or hydroxylamine or other possible intermediates such as hyponitrite are present in appreciable concentration as free intermediates in the cell. It is significant that, in a total of 45 non-nitrite-utilizing mutants, none has been found that could represent an independent structural gene for nitrite reductase, hyponitrite reductase or hydroxylamine reductase. The *niiA* mutants possess inducible nitrate reductase and lack both nitrite reductase and hydroxylamine reductase. When *niiA* mutants are grown on urea and nitrate, nitrite is excreted into the medium. The prototroph and the rest of the mutants do not excrete nitrite under the same conditions (J. A. Pateman, unpublished work). It may be that nitrite is reduced directly to ammonium on a single enzyme molecule, perhaps because the

free intermediates would be too unstable or toxic. It has been postulated that ammonium is the product of the reduction of nitrite by nitrite reductase without the formation of free intermediates in various higher plants (Joy & Hageman, 1966; Ingle, Joy & Hageman, 1966). The *niiA* locus may be the structural gene for such an enzyme in *Aspergillus*.

The *niiB* mutants possess nitrate-reductase, nitrite-reductase and hydroxylamine-reductase activities similar to those of the uninduced prototroph even when grown in the presence of nitrate. There are a number of reasons why mutation at a single gene locus could result in a decrease of three enzyme activities. The main possibilities are outlined below together with the available evidence that bears on them.

(1) The *niiB* locus might determine a permease system responsible for the uptake of the inducing substrates. However, it has been shown by studies of nitrite toxicity that the *niiB* locus cannot be responsible for a nitrate permease or nitrite permease (J. A. Pateman, unpublished work).

(2) The *niiB* locus might determine some common factor necessary for all three enzyme activities. This common factor could be of several kinds: (a) It might simply be a specific kind of cofactor or prosthetic group for the three enzymes. The only likely candidate for such a role is a specific form of flavine. It is not possible to supplement the growth of *niiB* mutants on nitrate or nitrite by the addition of a flavine. However, another functionally distinct type of mutant, *niiB* *riboA*, does respond to flavine under these conditions. The *niiB* *riboA* gene therefore appears to be involved in the synthesis of such a cofactor, and it is possible then that the *niiB* gene is also involved. Future work should clarify this problem if the *niiB* gene should prove not to be a regulator gene (see hypothesis 3 below). (b) A second possibility is that NADPH is not the actual electron donor for these three enzymes in *Aspergillus*. It has been reported that an NADPH-ferredoxin reductase is present in higher plants that can transfer electrons from NADPH to suitable electron acceptors such as ferredoxin or benzyl viologen (Ingle *et al.* 1966). These authors suggest that reduced ferredoxin is the natural electron donor for nitrite reductase in higher plants and showed that reduced benzyl viologen would act as an electron donor for nitrite reductase *in vitro*. Also, Sorger (1965) has shown in *Neurospora* that NADPH-nitrate reductase has benzyl viologen-nitrate-reductase activity. If the three nitrate-induced enzymes in *Aspergillus* could *in vivo* use only reduced ferredoxin as an electron donor then a functional NADPH-ferredoxin reductase might be essential for the reduction of nitrate to ammonium. Mutation in a structural gene for such an NADPH-

ferredoxin reductase would result in the properties of the *niiB* strains. Further, such a mutant should still possess the enzymes necessary to reduce nitrate, nitrite and hydroxylamine with reduced ferredoxin or reduced benzyl viologen as an electron donor. Accordingly the prototrophic strain and representatives of all the types of mutant were assayed for benzyl viologen-nitrate reductase. Table 4 shows that the prototroph and mutant *niiA*, both of which possess inducible NADPH-nitrate reductase, also possess nitrate-inducible benzyl viologen-nitrate reductase. All the mutants that lack NADPH-nitrate reductase also lack benzyl viologen-nitrate reductase, including 12 independently mutated *niiB* strains. Therefore it is improbable that the *niiB* locus is a structural gene for an NADPH-ferredoxin reductase that is essential for the reduction of nitrate. (c) A third possibility is that *in vivo* in *Aspergillus* the reduction of nitrate to ammonium is carried out on a single molecule that consists of a number of polypeptide sub-units. The existence of such an aggregate, termed a 'nitrosome', has been postulated in *Candida utilis* (A. H. Bussey & A. P. Sims, personal communication). If such a 'nitrosome' existed in *Aspergillus* the *niiB* gene might determine a structural polypeptide essential for the stability and function *in vivo* of the 'nitrosome'. Mutation in such a gene could easily result in the properties of the *niiB* strains. Future work on the purification and properties of the *Aspergillus* enzymes should clarify this problem if the *niiB* gene should prove not to be a regulator gene (see hypothesis 3 below).

(3) The *niiB* locus may be responsible for the production of some component of the regulatory system for the nitrate-reduction pathway. A number of examples are known of mutants that lack several enzyme activities and, like *niiB* strains, are recessive in heterocaryons or heterozygous diploids. The *C* gene in the L-arabinose system in *Escherichia coli* (Englesberg, Irr, Power & Lee, 1965) and the *uaY* mutants in *Aspergillus*, which lack xanthine-dehydrogenase and urate-oxidase activities (Darlington, Scazzocchio & Pateman,

1965; Scazzocchio, 1966), are examples. Results have been obtained in this Laboratory that support this hypothesis (J. A. Pateman & D. J. Cove, unpublished work).

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