GENETIC CONTROL OF NITRATE REDUCTASE IN *NEUROSPORA CRASSA*[1]

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IN *Neurospora crassa* nitrate reduction is catalyzed by an electron transport system in which TPNH (reduced triphosphopyridine nucleotide) donates electrons to FAD (flavine adenine dinucleotide) *in vitro*. Reduced FAD in turn passes the electrons on to either cytochrome c or to molybdate, and reduced molybdate then passes the electrons on to nitrate, which is thereby converted into nitrite and water. Like other systems of its kind, the above electron transport sequence has been characterized biochemically as follows: (a) by determining what cofactors are required by dialyzed extracts to reduce nitrate enzymatically with TPNH (NASON and EVANS 1953; NICHOLAS and NASON 1954; KINSKY and MCELROY 1958; NICHOLAS 1961); (b) by inhibiting the redox sequence with compounds known to attack or to compete with these cofactors (NICHOLAS and NASON 1954; NASON 1956); (c) by partial purification of the total electron transport sequence and subsequent isolation and characterization of some of its components (EVANS and HALL 1955); and finally, (d) by the use of several reducing agents as electron donors for nitrate reduction (EVANS and NASON 1952; NICHOLAS and NASON 1954). The nitrate reductase system has been shown to be adaptive to nitrate (NASON and EVANS 1953; KINSKY 1961) and inducible (SORGER 1965) in Neurospora, and in *Escherichia coli* (POLLOCK 1946; WAINWRIGHT and POLLOCK 1949; WAINWRIGHT and NEVILL 1956 a, b). At least one of the steps in the electron transport sequence from TPNH to nitrate in Neurospora nitrate reductase (measured as nitrate-inducible TPNH cytochrome c reductase) has also been demonstrated to be inducible by nitrate (KINSKY and MCELROY 1958; SORGER 1965) and to remain physically associated with the rest of the electron transport chain during a 50- to 70-fold purification (KINSKY and MCELROY 1958) and during sucrose density gradient centrifugation (SORGER 1963).

A comprehensive combined genetic and biochemical study on Neurospora nitrate reductase has been initiated in an effort to answer the following questions: How many polypeptides or enzymes are required to carry out nitrate reduction? Must these polypeptides or enzymes be organized or aggregated in order to catalyze this reduction, and if so how? And in what way is the electron transport sequence from TPNH to nitrate controlled metabolically?

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Nitrate reductase in Neurospora has certain unique advantages for such a study: (a) the electron transport sequence is obligately required by the mold for growth on nitrate but not for growth on nitrite or ammonia; therefore, unlike many other systems of this general type, mutants lacking a functional nitrate reductase electron transport system can be found relatively easily and maintained on ammonia; (b) the electron transport sequence from TPNH to nitrate can be measured easily at at least one intermediate step, i.e., cytochrome c reduction; (c) the chromosomes of Neurospora are relatively well mapped genetically.

Nitrate reductase-less mutants have been found in Neurospora (DE LA HABA 1950; SILVER and MCELROY 1954), but no genetic studies were performed with them. On the other hand, mutants at four different loci which cannot utilize nitrate as a nitrogen source have been described. One of these mutants (*nit-4*) (BLAKELY and SRB 1962) will not grow on nitrite as a nitrogen source, and hence is probably blocked beyond nitrate reductase in the reductive assimilation of nitrate. The mutants at the other three loci (*nit-1*, *nit-2*, *nit-3*) (HOULAHAN, BEADLE, and CALHOUN 1949; PERKINS 1959), had not been characterized biochemically. The earliest reports of combined genetic and biochemical studies on Neurospora (SORGER 1963); Aspergillus (COVE and PATEMAN 1963), and corn (HAGEMAN, ZIESERL, and LENG 1963) indicated that nitrate reductase was controlled by more than one gene.

The two principal objectives of the present investigation were: (a) To establish a link between the genetic studies on Neurospora gene loci involved in the control of nitrate utilization and the biochemical investigations on nitrate reductase in wild type and in previously unmapped nitrate reductaseless Neurospora strains. This immediate objective is part of a larger attempt to answer the question: How many genes are involved in the control of nitrate reduction? (b) To investigate the nature of the function controlled by each gene.

MATERIALS AND METHODS

Strains: Neurospora crassa strains 74A and 3.1a were used as wild types. The mutants *pyr-1* and *pyr-2* were obtained from the Fungal Genetics Stock Center at Dartmouth College. Strains *ad-4* (F45), *ad-4* (F48), and *pan-1* were from the mutant collection at Yale University.

Mutants *nit-1* (34547), *nit-2* (K31), and *nit-3* (14789) were obtained from the Fungal Genetics Stock Center at Dartmouth College and backcrossed at least once to 74A or 3.1a wild types.

Media: The *Basic Medium* contained the following in grams per liter: sodium potassium tartrate, 1.0; KH_2PO_4, 2.0; $NaH_2PO_4 \cdot 2H_2O$, 0.8; $MgSO_4 \cdot 7H_2O$, 0.5; NaCl, 0.1; $CaCl_2$, 0.1; sucrose, 20.0; biotin 5×10^{-6}; Fries Trace Element solution, 1 ml per liter (RYAN, BEADLE, and TATUM 1943). *Ammonia Medium* consisted of *Basic Medium* plus 4.0 g NH_4Cl per liter as nitrogen source. *Minimal Nitrate Medium* was composed of *Basic Medium* and 5.0 g $NaNO_3$ per liter as nitrogen source. Occasionally $NaNO_2$ was used as a nitrogen source at a concentration of 0.5 g/l, and the medium was then adjusted to pH 6.5. Purine and pyrimidine mutants were maintained on Fries medium (RYAN, BEADLE, and TATUM 1943) plus 100 μg/ml of their respective supplements, and *pan-1* mutants on a Fries medium plus 30 μg/ml calcium pantothenate. Plating media contained the same macro- and micronutrients as described above with the exception of the carbon source, which was replaced by 1 g glucose and 10 g sorbose per liter to induce colonialization, and 15 g agar per liter to solidify.

Mutagenic treatment: Conidia were irradiated with approximately 5000 ergs of ultraviolet

light from a 15 w germicidal lamp essentially as described by GILES (1951), and subsequently allowed to remain in darkness for 30 minutes to prevent photoreactivation. Surviving conidia were then subjected to fluoride (MEGNET 1964) or filtration concentration (WOODWARD, DEZEEUW, and SRB 1954) techniques. The ungerminated, surviving conidia were subsequently plated on *Ammonia Medium*. Colonies were isolated onto solid *Ammonia Medium* and on *Nitrate Medium*. Mutants were designated as *nr* if they grew on *Ammonia* and on *Nitrite* but not on *Minimal Nitrate Medium*. Mutants unable to use nitrate as a nitrogen source are termed nitrate mutants, symbolized *nit*.

Crossing and complementation: The methods used are described in RESULTS.

Induction: Mycelial pads were grown from a conidial inoculum in a liter of *Ammonia Medium* for 6 to 10 days in standing culture in 2.5 l fernbach flasks at 25°C in the dark. Pads were then harvested with cheesecloth, washed twice with distilled water, and cut into sectors. The experimental (induced) sectors were placed in 1 l fernbach flasks containing 250 ml of *Minimal Nitrate Medium*, the control (uninduced) sectors in 250 ml of *Basic Medium* containing no nitrogen source. All sectors were then incubated on a rotary shaker in the dark at 25°C for 16 to 18 hours. When sectors were induced on a medium containing NH_4Cl as nitrogen source, no nitrate reductase or induced TPNH cytochrome c reductase activity was detected in the crude extract so *Basic Medium* was used for induction of the "uninduced" sectors (SORGER 1963b).

Extraction of mycelium: At the end of the induction period, the mycelial pads were harvested with cheesecloth, washed twice with distilled water, and blotted between paper towels until no more moisture could be squeezed out by the application of manual pressure. The blotted pads of mycelium were immersed in liquid nitrogen for no less than 10 minutes and subsequently ground in an ice-cold mortar with about a half weight of acid-washed powdered glass. The resulting paste was then suspended evenly in ten volumes of cold 0.1M potassium phosphate buffer pH 7.0, and incubated 30 minutes in the cold. At this time the slurry was centrifuged at 20,000 *g* for 30 minutes in a refrigerated Spinco model L centrifuge, and the supernatant used immediately.

Assay for nitrate reductase: The assay mixture contained the following as final concentrations in 0.60 ml: TPNH, 1.67×10^{-4} M; FAD, 8.3×10^{-7} M; sodium pyrophosphate buffer pH 7.0, 6.7×10^{-2} M; KNO_3, 1.67×10^{-2} M; and extract, 0.20 ml. The mixture was allowed to incubate at room temperature for 15 minutes and the reaction stopped by the addition of 0.1 ml 25.5% barium acetate, which precipitates the pyridine nucleotides, followed by 2.5 ml of 95% ethanol to precipitate the proteins. The resulting suspension was then centrifuged in a desk model Spinco centrifuge at approximately 4000 g for 5 minutes and the clear supernatant saved for colorimetric analysis. Nitrite was assayed in this supernatant by adding 1 ml each of 1% sulfanilamide (Eastman) in 3.2M HCl, and 0.02% aqueous N-1 naphthyl ethylene diamine dihydrochloride (Eastman) and allowing the mixture to incubate at room temperature for 30 minutes. One unit of activity is defined as a difference in absorbancy at 540 mμ of 0.10 optical density (O.D.) units in a 1 cm light path (in a Beckman DK-2 recording spectrophotometer) between a sample incubated with TPNH and one incubated without. One unit of activity is equivalent to the production of 10 mμmoles of nitrite. Specific activity is expressed as units of activity per mg of protein. Protein was measured by the biuret test (DAWSON, ELLIOTT, ELLIOTT and JONES 1959). All measurements of activity were made in a range of enzyme concentration where the ratio of activity to enzyme concentration was constant.

Assay of TPNH cytochrome c reductase: The assay mixture contained the following in final concentrations in a volume of 0.70 ml: TPNH, 2.28×10^{-4}M; FAD, 4.3×10^{-6}M; cytochrome *c* (Sigma type III), 1.16×10^{-4}M; potassium phosphate buffer pH 7.0, 7.1×10^{-2}M; and extract, 0.1 ml. A final concentration of 8.3×10^{-4}M KCN was included in the assay mixture containing crude extract, but cyanide was omitted when assaying purified preparations. Assays were conducted by determining the rate of increase in concentration of reduced cytochrome c during the first 20% of the reaction. One unit of activity is defined as an increase of 0.01 O.D. units at 550 mμ per minute at 24 ± 2°C in a 1 cm light path in a Beckman DK-2 recording spectrophotometer. In all experiments the reference cuvette contained all reactants except TPNH. Again, all measurements of activity were made in that range of enzyme concentration where the ratio of activity to enzyme concentration was constant.

RESULTS

(1) *Induction of new mutants:* The nitrate reductase-less mutants isolated during this study had the following properties after at least one backcross to wild type: growth and conidiation like wild type on ammonia, on nitrite, or on a protein hydrolysate as alternative nitrogen sources; no detectable growth on nitrate after five days incubation at 25°C in a liquid medium; no nitrite accumulation when grown from a conidial inoculum to a felt on high nitrate-containing liquid Fries medium (which contains ammonia-nitrogen); 1:1 ratio of mutant to wild-type ascospores among the progeny from a backcross of a mutant to wild type; the capacity to form a heterokaryon with an adenine mutant in a 74A wild-type genetic background.

Table 1 indicates the origin of the 49 mutants answering this description which were isolated and retained in this study. In all, 70 mutants were isolated originally, but 21 of these were discarded because they did not possess the properties mentioned above and/or could not be backcrossed to wild type.

(2) *Genetic relationships between newly isolated mutants and* nit-1, nit-2, *and* nit-3: Each newly isolated mutant was crossed to each of the previously mapped stock collection mutants by the procedure described below.

Crosses were made on cornmeal agar (Difco), and allowed to mature from 4 to 7 weeks before harvesting. Single random ascospores were isolated onto agar blocks which were subsequently introduced into tubes of *Ammonia Medium*, heat shocked 40 min at 60°C, and incubated at 25°C in a growth chamber until conidiation. Conidia from these cultures were then tested for growth on liquid *Ammonia Medium* versus liquid *Minimal Nitrate Medium.* The above painstaking procedure was adopted after much testing because it avoids the following problems: (a) *nit* mutants grow, although poorly, on solid *Minimal Nitrate Medium*, whereas they show essentially no growth on liquid *Minimal Nitrate Medium.* Thus, the use of growth tests in liquid medium avoids possible confusion in the phenotypic classification of these mutants. (b) Isolation of individual ascospores eliminates the possibility of a crowding effect which might result in the masking of the wild-type phenotype (GRIGG 1952). (c) The long maturation time utilized served to minimize the possible problem of differential ascospore maturation and viability. Ascospore germination in most crosses was better than 85%. Furthermore, microscopic inspection of

TABLE 1

Nitrate reductaseless mutants collected by filtration concentration and fluoride techniques

	Mutant hunt No.				
	1	2	3	4	5
Strain used (Wild type)	*74A*	*3.1a*	*74A*	*74A*	*74A*
Mutagen used	None	UV*	UV	UV	UV
Initial number of conidia ($\times 10^{-6}$)	100	106	100	104	104
Number of colonies examined from plated filtration concentrate	97	696	524	873	721
Filtration time (hours)	80	60	60	84	...
Fluoride time (hours)	..	..	..	..	100
No. nitrate nonutilizers	1	15	1	24	8
No. nitrite accumulators (not kept)	0	2	0	2	0

* 33% survival; M. E. CASE, personal communication.

the ascospores both inside and out of the perithecia showed that the vast majority of the spores were black (ripe) before a cross was plated.

Tests for pseudowild ascospores were made by plating conidia from presumptive wild-type isolates onto *Ammonia Medium*, isolating 50 colonies onto *Ammonia Medium*, and testing these on liquid *Minimal Nitrate*. No pseudowild ascospores were detected in the crosses listed in Table 2.

Thirty-one mutants were successfully crossed to *nit-1*; the average germination in these crosses was 93.2%, and the average recombination between the new mutants and *nit-1* was 43 ± 8%. Thirty-eight mutants (excluding nr 15) were crossed successfully to *nit-3*. The average germination was 88.4%, and the average recombination between the new mutants and *nit-3* was 53 ± 8%.

The results indicate that 30 of the 31 newly isolated mutants shown in Table 2

TABLE 2

Recombination between nit-2 *and newly isolated mutants*

Mutant isolation numbers	Average recombination percent	95 percent confidence interval (percent recomb.)	Average germination percent	No. ascospores examined	No. crosses made
nr 1	1	0–10	95.0	100	1
nr 2	0	0–6	96.5	200	2
nr 6	0	0–2	86.3	300	3
nr 7	0	0–8	97.0	100	1
nr 10	0	0–1	81.7	700	7
nr 15	50	42–62	87.2	400	4
nr 16	0	0–8	81.0	100	1
nr 17	0	0–2	87.0	300	3
nr 18	0	0–8	92.0	100	1
nr 20	0	0–8	88.0	100	1
nr 21	0	0–14	56.0	100	1
nr 22	0	0–6	95.0	200	2
nr 23	0	0–1	96.0	300	3
nr 24	0	0–2	83.0	300	3
nr 25	0	0–6	91.5	200	2
nr 26	0	0–8	95.0	100	1
nr 27	0	0–8	69.0	200	2
nr 28	0	0–1	82.2	500	5
nr 29	0	0–8	95.0	100	1
nr 30	0	0–2	78.3	300	3
nr 31	0	0–1	81.2	600	6
nr 33	0	0–1	84.2	500	5
nr 34	0	0–8	100.0	100	1
nr 36	0	0–8	98.0	100	1
nr 37	0	0–8	96.0	100	1
nr 38	0	0–10	68.0	100	1
nr 39	0	0–8	95.0	100	1
nr 40	0	0–14	49.0	100	1
nr 41	0	0–6	74.5	200	2
nr 43	0	0–8	99.0	100	1
nr 45	0	0–8	95.0	100	1
(*nit-1*)	44	28–62	96.0	100	1
(*nit-3*)	58	30–82	94.0	113	1

The percent recombination is twice the observed percent of prototrophs among the progeny.

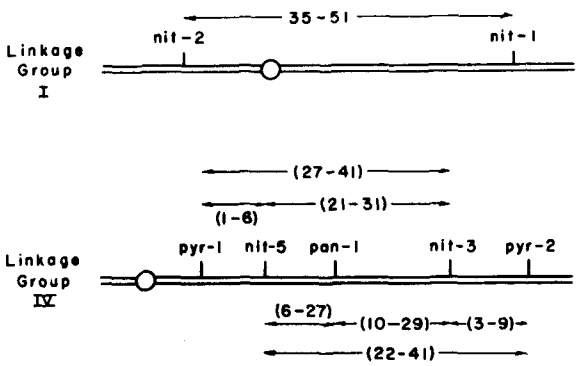

FIGURE 1.—Location of *nit* genes. Crosses were performed as described in text. The germination in these experiments was 81 ± 13%. 95% confidence intervals are shown in parenthesis.

(i.e., all with the exception of nr 15) are allelic to *nit-2*. nr 15 exhibited relatively high recombination with all three stock collection mutants, and thus constitutes a new *nit* locus, which, to fit the existing nomenclature, is being designated *nit-5*. The new gene was mapped by crossing nr 15 to several other mutants on linkage group IV. The results are summarized in Figure 1. On the basis of available two-point cross data, *nit-5* appears to be located on the right arm of linkage group IV between *pyr-1* and *pan-1*. The *nit-3* and *nit-4* loci are also located on this arm, the latter being approximately 15 crossover units to the right of *cot*, which is near *nit-3*.

(3) *Complementation studies:* In order to determine the functional relationships of the various *nit* mutants, heterokaryon complementation tests were performed. In these tests, two forcing markers were employed in order to insure heterokaryon formation. The following procedure was adopted in performing the heterokaryon tests. Each *nit* mutant used in the tests was crossed to each of two adenine mutants (*ad-4* F45 and *ad-4* F48) which were known to complement well with each other, and to form heterokaryons with a stable nuclear ratio close to unity (WOODWARD 1959). Double mutant (*nit ad-4* F-x) progeny from these crosses were selected on the basis of the rapidity with which they formed heterokaryons with *ad-4* F-y testers on solid Fries medium. Backcross isolates that took longer than 24 hours to form a heterokaryon with the tester were discarded or backcrossed once more. At least two isolates of each of the following genetic constitutions were kept: *ad-4* F45*A nit-x*, *ad-4* F45*a nit-x*, *ad-4* F48*A nit-x* and *ad-4* F48*a nit-x*. Conidial suspensions of approximately equal optical densities were made from each *nit-x* F45 and from each *nit-y* F48. Aliquots of these suspensions were mixed in all possible pairwise combinations between *nit-x* F45 and *nit-y* F48 in liquid *Ammonia Medium*. In instances of positive responses, heterokaryons had formed sizeable mycelial growth within 72 hours, and by 96 hours they had conidiated. Conidia from these heterokaryons were then tested for growth on *Minimal Nitrate* and *Ammonia* liquid medium. Complementation was recorded as positive if growth occurred on both types of media, and as negative

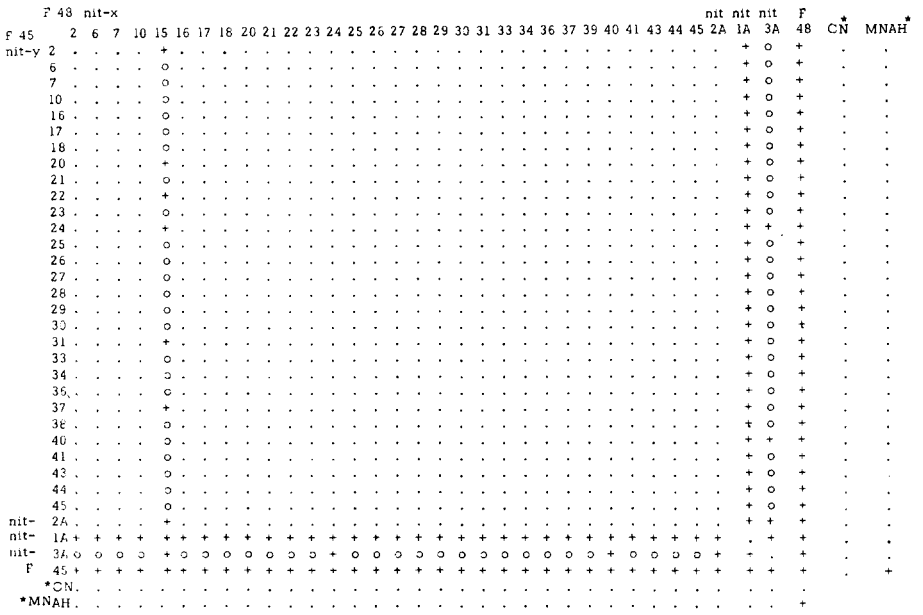

FIGURE 2.—Complementation among *nit* mutants. A heterokaryon between each F45 *nit-y* (*ad-4* nr y) and each F48 *nit-x* (*ad-4* nr x) was performed on *Ammonia Medium* without added adenine. Conidia from this preformed heterokaryon were then tested on *Minimal Nitrate Medium*, which also lacks adenine. These results refer to the response of conidia from preformed heterokaryons. + = growth on minimal nitrate; . = no growth on minimal nitrate; 0 = test not done. CN = Fries medium; MNAH = minimal nitrate+adenine+histidine. * Controls: media inoculated with conidia from one strain only.

if it only occurred on *Ammonia Medium*. Each pairwise test was repeated at least twice with at least two different backcross isolates of each double mutant.

As part of each complementation experiment, conidia from each *nit-x* F48 isolate were mixed in *Ammonia* (adenineless) liquid medium with conidia from *nit*$^+$ F45. Conidia from each *nit-y* F45 were tested similarly with conidia from *nit*$^+$ F48. When tested, the resulting heterokaryons grew on *Minimal Nitrate Medium* indicating that all the *nit* mutations tested were recessive.

The results of the heterokaryon tests (Figure 2) indicate that nitrate reductase mutants comprise four distinct functional groups (genes or cistrons) corresponding to the four loci controlling nitrate utilization. In the sample of *nit-2* alleles tested, there was no evidence of allelic complementation.

Crude extracts of various heterokaryons were tested for the presence of nitrate reductase and inducible TPNH cytochrome c reductase. All heterokaryons exhibiting a positive complementation response had both activities.

(4) *Biochemical characterization of* nit *mutants:* In order to investigate the nature of the function controlled by each of the four *nit* genes, mycelial extracts of mutants of each type were assayed for nitrate reductase and for nitrate-inducible TPNH cytochrome c reductase. The results of these tests (Table 3)

TABLE 3

Biochemical characterization of nit *mutants*

Strain	N source in induction medium	Mean sp. ac. TPNH cytochrome c reductase (CR)*	induced CR/ uninduced CR	Mean sp. ac. nitrate reductase (NR)*	Nitrate reductase inhibitor
Wild type	nitrate	79	5.6 ± 1.2	6.0 ± 1.0	—
	none	17		0	—
nit-1	nitrate	77	2.0 ± 0.1	0	—
	none	34		0	
nit-2	nitrate	18	0.9 ± 0.0	0	—
	none	20		0	
nit-3	nitrate	17	0.9 ± 0.1	0	+
	none	15		0	
nit-5	nitrate	21	1.2 ± 0.6	0	—
	none	15		0	

Mycelia were induced as described in METHODS. + indicates the presence of an inhibitor; − indicates its absence.
* Based on at least five experiments.

show that extracts from induced mycelia of *nit-1*, *nit-2*, *nit-3*, and *nit-5* lack detectable nitrate reductase and that only *nit-1* retains the ability to form a nitrate-inducible TPNH cytochrome c reductase. The following observations indicate that the above results are not due to an altered permeation mechanism: Aliquots from conidial suspensions of each of the mutants studied were dispensed into flasks containing 100 ml of *Ammonia Medium* plus 0, 0.2, 0.6, or 2.0 mg sodium molybdate added per ml. The inoculated flasks were then incubated for 7 days at 25°C in standing culture, and subsequently the mycelial pads were harvested, dried to constant weight in an 80°C oven, and weighed. The dry weights of the mycelial pads cultured in the presence of 0, 0.2, and 0.6 mg molybdate per ml were no different from each other, while those cultured in the presence of 2.0 mg molybdate per ml were at least 40% lighter than their counterparts grown without the added metal salt. This indicates that molybdate can poison *nit* mutants as well as wild-type mycelium, therefore it must be getting into the cells of all these strains. These same mutants were tested for growth on *Minimal Nitrate Medium* to which 50 g/l potassium nitrate or 0.6 mg/ml sodium molybdate had been added. Wild type grew on this medium, but none of the *nit* mutants tested did. When a mycelial pad from a representative mutant at each locus was induced with nitrite (on which all mutants grow, by definition), or in the presence of a sublethal concentration of sodium molybdate (0.6 mg/ml), no change in the above pattern was observed regarding the presence of the inducible activities in extracts.

Tests for the presence of possible inhibitors were performed by mixing equal volumes of crude extracts from induced wild-type mycelium with crude extracts

from mycelia of each mutant. Each such test was performed at least three times with different extracts. The results summarized in Table 3 show that only in one induced mutant extract, that of *nit-3*, was an inhibitor of nitrate reductase detected. A statistical analysis of these results indicated that the inhibition was significant ($P < 0.01$ that the observed effect was due to random variation). None of the extracts contained a detectable TPNH cytochrome c reductase inhibitor.

Finally, all mutants allelic to *nit-2* either did not grow or grew very poorly on hypoxanthine as a nitrogen source, whereas *nit-1*, *nit-3*, and *nit-5* mutants grew as well as wild type under these same conditions.

DISCUSSION

The first principal objective of this investigation was to establish a link between the genetic studies on loci involved in the control of nitrate utilization and the biochemical investigations on nitrate reductase in wild type and in previously unmapped nitrate reductase-less strains. This has been accomplished. The previously known loci, *nit-1*, *nit-2*, *nit-3*, and the newly discovered locus *nit-5*, all control nitrate reduction. The broader question of how many genes control nitrate reduction in Neurospora has been only partially answered. There must be at least four.

It may seem somewhat surprising that most of the newly isolated mutants were allelic to *nit-2*. However, all the auxotrophs found in this study were isolated by the filtration-concentration and fluoride techniques, which are quite different from the total isolation procedure used to obtain the original *nit* mutants.

The following observations indicate that none of the four loci control a molybdate or a nitrate permease: all the mutants tested were able to use nitrite as a nitrogen source, but were not induced by nitrite or by high levels of nitrate; every mutant tested was sensitive to poisoning by the same concentration of molybdate in the growth medium, but none of these mutants would grow on *Minimal Nitrate Medium* in the presence of high but sublethal levels of molybdate, nor could any of the mutants tested be induced by nitrate in the presence of high but sublethal levels of this metal salt.

The second objective of this study—to investigate the nature of the function controlled by each gene—has been partially accomplished. Present evidence indicates that all four loci lack detectable nitrate reductase activity, but that the *nit-1* mutant retains activity for a nitrate-inducible TPNH cytochrome c reductase.

The following hypothesis appears to account for the observations reported here as well as for some recent findings on *nit-1* (SORGER 1964): nitrate reductase in its active form consists of an aggregate of at least two polypeptides, the first of which catalyzes the transport of electrons from TPNH to FAD and from FAD to cytochrome c (measured as TPNH cytochrome c reductase), and the second of which catalyzes the acceptance by molybdate of electrons from FAD and the subsequent transfer of these electrons to nitrate (measured as nitrate reductase when in the presence of active nitrate-inducible TPNH cytochrome c reductase).

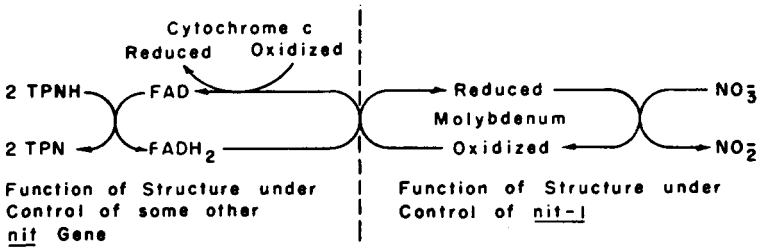

FIGURE 3.—Functional model of nitrate reductase in Neurospora.

This hypothesis is illustrated in Figure 3. On the basis of this model, mutants at *nit-1* have a mutationally altered second polypeptide and, hence, retain the ability to form active inducible TPNH cytochrome c reductase but lack the ability to form active nitrate reductase; mutants at some other *nit* locus, controlling the first polypeptide, would lack the ability to form both the cytochrome c reductase and nitrate reductase. This simple hypothesis does not account for all the observations on *nit-2* and *nit-3* mutants, but further detailed speculation is not warranted at this time. It is also worthy of note, but difficult to explain at present, that TPNH cytochrome c reductase in *nit-1* is partially constitutive.

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SUMMARY

Combined recombination and complementation analyses show that at least four loci (*nit-1*, *nit-2*, *nit-3*, and *nit-5*) control the electron transport sequence from TPNH to nitrate in nitrate reductase of Neurospora. *nit-5* is newly discovered, and is located in linkage group IV.

Extracts of mycelia from mutants at all four loci have no detectable nitrate reductase. *nit-1* mutants retain the ability to form nitrate-inducible TPNH cytochrome c reductase; mutants at the other loci have lost this ability. Probably none of these *nit* loci controls a nitrate or a molybdate permease.

LITERATURE CITED

BLAKELY, R. M., and A. M. SRB, 1962 Studies of the genetics and physiology of a nitrate non-utilizing strain of Neurospora. Neurospora Newsletter **2**: 5–6.

COVE, D. J., and J. A. PATEMAN, 1963 Independently segregating genetic loci concerned with nitrate reductase in *Aspergillus nidulans*. Nature **198**: 262–263.

DAWSON, R. M. C., D. C. ELLIOTT, W. H. ELLIOTT, and K. M. JONES, 1959 *Data for Biochemical Research*, Clarendon Press, Oxford.

DE LA HABA, G., 1950 Studies on the mechanism of nitrate assimilation in Neurospora. Science **112**: 203–204.

EVANS, H. J., and N. S. HALL, 1955 Association of molybdenum with nitrate reductase from soybean leaves. Science **122**: 922–923.

EVANS, H. J., and A. NASON, 1952 The effect of reduced TPN on nitrate reduction by purified nitrate reductase. Arch. Biochem. Biophys. **39**: 234–235.

GILES, N. H., 1951 Studies on the mechanism of reversion in biochemical mutants of *Neurospora crassa*. Cold Spring Harbor Symp. Quant. Biol. **16**: 283–313.

GRIGG, G. W., 1952 Back mutation assay method in microorganisms. Nature **169**: 98–100.

HAGEMAN, R. H., J. F. ZIESERL, and E. R. LENG, 1963 Levels of nitrate reductase activity in inbred lines and F_1 hybrids in maize. Nature **197**: 263–265.

HOULAHAN, M. B., G. W. BEADLE, and H. G. CALHOUN, 1949 Linkage studies with biochemical mutants of *Neurospora crassa*. Genetics **34**: 493–507.

KINSKY, S., 1961 Induction and repression of nitrate reductase in *Neurospora crassa*. J. Bacteriol. **82**: 898–904.

KINSKY, S., and W. D. MCELROY, 1958 Neurospora nitrate reductase: The role of phosphate, flavine and cytochrome c reductase. Arch. Biochem. Biophys. **73**: 466–483.

KLEIN, D. T., 1960 Interrelations between growth rate and nuclear ratios in heterokaryons of *Neurospora crassa*. Mycologia **52**: 137–147.

MEGNET, R., 1964 A method for the selection of auxotrophic mutants of the yeast *Schizosaccharomyces pombe*. Experientia **20**: 320–321.

NASON, A., 1956 Enzymatic steps in the assimilation of nitrate and nitrite in fungi and green plants. pp. 109–136. *A Symposium on Inorganic Nitrogen Metabolism*. Edited by W. D. MCELROY and B. GLASS. Johns Hopkins Press, Baltimore.

NASON, A., and H. J. EVANS, 1953 Triphosphopyridine nucleotide-nitrate reductase in Neurospora. J. Biol. Chem. **202**: 655–673.

NICHOLAS, D. J. D., 1961 Minor mineral nutrients. Ann. Rev. Plant Physiol. **12**: 63–90.

NICHOLAS, D. J. D., and A. NASON, 1954 Mechanism of action of nitrate reductase from Neurospora. J. Biol. Chem. **211**: 183–197.

NICHOLAS, D. J. D., A. NASON, and W. D. MCELROY, 1954 Molybdenum and nitrate reductase: II. Molybdenum as a constituent of nitrate reductase. J. Biol. Chem. **207**: 353–360.

PATEMAN, J. A., D. J. COVE, B. M. REVER, and D. B. ROBERTS, 1964 A common cofactor for nitrate reductase and xanthine dehydrogenase which also regulates the synthesis of nitrate reductase. Nature **201**: 58–60.

PERKINS, D. D., 1959 New markers and multiple point linkage data in Neurospora. Genetics **44**: 1185–1208.

POLLOCK, M. R., 1946 Adaptation of "nitratase" in washed suspensions of bacteria. Brit. J. Exptl. Pathol. **27**: 419–432.

RYAN, F. J., G. W. BEADLE, and E. L. TATUM, 1943 The tube method of measuring the growth rate of Neurospora. Am. J. Botany **30**: 784–799.

SILVER, W. S., and W. D. MCELROY, 1954 Enzyme studies on nitrate and nitrite mutants of Neurospora. Arch. Biochem. Biophys. **51**: 379–394.

SORGER, G. J., 1963 The genetic control of nitrate reductase in *Neurospora crassa*. (Abstr.) Genetics **48**: 911. —— 1963 TPNH cytochrome c reductase and nitrate reductase in

mutant and wild-type Neurospora and Aspergillus. Biochem. Biophys. Res. Commun. **12**: 395–401. —— 1964 Use of mutants to study the electron transport sequence of nitrate reductase. Nature **204**: 575–576. —— 1965 Simultaneous induction and repression of nitrate reductase and TPNH cytochrome c reductase in *Neurospora crassa*. Biochim. Biophys. Acta **99**: 234–245.

WAINWRIGHT, S. D., and A. NEVILL, 1956a The induced formation of nitrate reductase in auxotrophic mutants of *E. coli*. J. Bacteriol. **71**: 254–255. —— 1956b The influence of depletion of nitrogenous reserves upon the phenomenon of induced enzyme biosynthesis in cells of *Escherichia coli*. J. Gen. Microbiol. **14**: 47–56.

WAINWRIGHT, S. D., and M. R. POLLOCK, 1949 Enzyme adaptation in bacteria: Fate of nitratase in nitrate-adapted cells grown in absence of substrate. Brit. J. Exper. Pathol. **30**: 190–198.

WOODWARD, D. O., 1959 A study of gene-enzyme relationships at the *ad-4* locus in *Neurospora crassa*. Ph.D. Thesis, Yale University.

WOODWARD, V. W., J. R. DEZEEUW, and A. M. SRB, 1954 The separation and isolation of particular biochemical mutants of Neurospora by differential germination of conidia followed by filtration and selective plating. Proc. Natl. Acad. Sci. U.S. **40**: 192–200.