Proceedings of the National Academy of Sciences
Vol. 65, No. 1, pp. 137–144, January 1970

Formation of Assimilatory Nitrate Reductase by *in vitro* Inter-Cistronic Complementation in *Neurospora crassa**

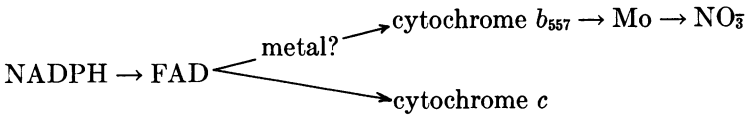
Alvin Nason, Alan D. Antoine,† Paul A. Ketchum,‡ William A. Frazier III, and David K. Lee

MCCOLLUM-PRATT INSTITUTE, THE JOHNS HOPKINS UNIVERSITY, BALTIMORE

Communicated by W. D. McElroy, September 25, 1969

Abstract. *In vitro* complementation of the soluble assimilatory nicotinamide adenine dinucleotide phosphate, reduced form (NADPH)-nitrate reductase was attained by mixing cell-free preparations of certain *Neurospora* nitrate reductase mutants: induced *nit-1* (uniquely possessing inducible NADPH-cytochrome *c* reductase) with (*a*) uninduced or induced *nit-2* or *nit-3*, or (*b*) uninduced wild type. The complementing activity of induced *nit-1* is soluble while that of *nit-2*, *nit-3*, and wild type is particulate but not of mitochondrial origin. All fractions are inactivated by heat or trypsin. The NADPH-nitrate reductase enzymes formed in the above three complementing mixtures are similar to the wild-type enzyme in sucrose density gradient profiles, molecular weight, substrate affinity, sensitivity to inhibitors and temperature, but show different ratios of associated enzyme activities. The data suggest that nitrate reductase consists of at least two protein subunits: a nitrate-inductible subunit as reflected by inductible NADPH-cytochrome *c* reductase, and a constitutive protein which is activated (as indicated by the appearance of flavine adenine dinucleotide, reduced form ($FADH_2$)- and reduced methyl viologen-nitrate reductase activities) when it combines with the inductible subunit.

The discovery of assimilatory NADPH-nitrate reductase (NADPH : nitrate oxidoreductase, E.C. 1.6.6.2.) from *Neurospora crassa* and elucidation of its several components including their sequence of action[1–7] have established the following electron transport pathway for the enzyme complex:



All activities known to be associated with the above nitrate reductase complex, namely NADPH-nitrate reductase, flavine adenine dinucleotide (FAD)-dependent NADPH-cytochrome *c* reductase (cytochrome *c*, however, is not a component of the enzyme), $FADH_2$-nitrate reductase and reduced methyl viologen (MVH)-nitrate reductase are induced concomitantly by nitrate. The four activities are present in constant proportions during a 500-fold purification of the enzyme and are represented by a single protein complex of molecular weight 228,000 presumably consisting of two or more intimately linked protein subunits.[7]

Cove and Pateman[8] concluded that at least six genes control the synthesis of

inducible NADPH-nitrate reductase in *Aspergillus nidulans* and suggested that the enzyme is made up of several "different polypeptide subunits." Sorger and Giles[9, 10] stated that at least four genes control nitrate reductase in *Neurospora* and postulated the enzyme to be an aggregate of two polypeptide chains: one responsible for the transport of electrons from NADPH to FAD (and thence to cytochrome *c*), and the other for electron transfer from reduced FAD via molybdenum to nitrate. Recent studies[11] have shown *in vitro* complementation of both a soluble form and a particulate form of a *respiratory* type NADH-nitrate reductase by mixing soluble extracts from two different chlorate-resistant mutants of *E. coli* K_{12}.

The present paper describes the *in vitro* complementation of a soluble *assimilatory*-type NADPH-nitrate reductase in mixtures of cell-free preparations from certain *Neurospora crassa* mutant and uninduced wild-type strains lacking nitrate reductase. A preliminary report of this work has appeared.[12]

Materials and Methods. *Neurospora crassa* wild-type STA 4 and mutant strains *nit-1*, *nit-2* (33) and *nit-3* (and other mutants§ used in preliminary studies) deficient in assimilatory NADPH-nitrate reductase were obtained from the Fungal Genetics Stock Center, Dartmouth College, Hanover, N.H. The *nit-1* and *nit-2* genes are located on opposite arms of linkage group I, and the *nit-3* gene on linkage group IV (cf. Sorger and Giles[10]). The strains were routinely maintained on Bactoagar slants containing Fries basal medium with ammonium chloride (4.2 gm/liter) as the sole nitrogen source. Mycelia were grown in a liquid medium of the same composition in Fernbach flasks[7] until the wet weight was about 15 gm/flask. The mats were washed with distilled water, and induced (10 gm wet weight in 800 ml Fries medium containing sodium nitrate, 6.9 gm/liter, as the sole nitrogen source) for 2–3 hr in shaking culture. Washed mycelia were homogenized in Ten Broeck tissue homogenizers with cold preparation buffer (0.1 *M* phosphate buffer, pH 7.3, containing 10^{-3} *M* 2-mercaptoethanol and 5×10^{-4} *M* ethylenediaminetetraacetate (EDTA)) in proportions of 1 gm wet weight per 3 ml buffer, and centrifuged in the cold at $20{,}000 \times g$ for 20 min; and the supernatant solution (designated as *crude extract*) was used without further treatment unless stated otherwise.

Sources of substrates, cofactors, and other chemicals have been cited.[7] NADPH-nitrate reductase, $FADH_2$-nitrate reductase, MVH-nitrate reductase, and NADPH-cytochrome *c* reductase activities were routinely determined as described.[7] In several instances, as indicated, FMN (final concentration 10^{-4} *M*) was used in place of FAD for the NADPH-nitrate reductase assay. Constitutive NADPH-cytochrome *c* reductase is the activity obtained when FAD is omitted from the reaction mixture. Inducible NADPH-cytochrome *c* reductase is the activity with added FAD, corrected for constitutive activity. One unit of nitrate reductase and cytochrome *c* reductase is defined as the formation of 1 nmole (MANomole) nitrite/10 min and the reduction of 1 nmole (MANomole) cytochrome *c*/1 min, respectively. Protein was determined according to the procedure of Lowry *et al.*[13] Sedimentation analyses were performed with the 15.5–33% sucrose density gradient technique of Martin and Ames[14] using crystalline yeast alcohol dehydrogenase (Sigma) as a marker.

Results. When equal weights of freshly harvested or frozen mycelia of *Neurospora* mutants deficient in NADPH-nitrate reductase were homogenized together in all possible paired combinations, NADPH-nitrate reductase activity appeared only in those pairs which included induced *nit-1*. Prior induction of the seven other mutants, was not required. Of particular interest was the finding that homogenization of uninduced wild type with nitrate-induced *nit-1* also resulted in the production of NADPH-nitrate reductase. The concentration of enzyme in the homogenized mixtures increased two- or threefold with

time at 4°, often reaching a maximal level (ca. 25 units/mg protein) within 10 hours. Accompanying the production of NADPH-nitrate reductase was the appearance or stimulation of its associated activities.

Similar results, but with about 20 to 50 per cent the yield of nitrate reductase, were achieved by incubating the crude extract of induced *nit-1* with any of the separately prepared extracts of the other nitrate reductase mutants or uninduced wild type (Table 1). Extracts of uninduced (as well as induced) *nit-2* and uninduced wild type both lack NADPH-nitrate reductase and its associated activities, and respond almost identically in the production of activity when incubated with the extract of induced *nit-1*. The *nit-3* crude extract, however, contains endogenous $FADH_2$- and MVH-nitrate reductase activities which are increased some 10- and 20-fold (and higher), by nitrate induction. They appear unchanged, however, when incubated with a crude extract of induced *nit-1*, a mixture that nevertheless produced NADPH-nitrate reductase. The crude extracts of induced *nit-1* and induced wild type are unique in their possession of inducible, FAD-dependent NADPH-cytochrome *c* reductase (and a pyridine nucleotide-nitrite reductase). The inducible NADPH-cytochrome *c* reductase of nearly all mixtures displaying reconstituted NADPH-nitrate reductase was mostly contributed by the extract of induced *nit-1* (Table 1). In the case of induced wild type plus *nit-1*, the level of this activity was additive. An additional 7 hours incubation of the above mixtures at 4° resulted in a one- to twofold increase in NADPH-nitrate reductase. The apparent enhancement of nitrate reductase activity with time is ascribed in part to the progressive inactivation of the nitrite reductase which is contributed solely by the crude extract of nitrate-induced *nit-1*. The average level of NADPH-nitrate reductase produced in the above crude mixtures was about 25–30 per cent of the activity

TABLE 1. *In vitro complementation of NADPH-nitrate reductase and associated activities in mixtures of crude extract of uninduced and induced strains of Neurospora crassa mutants and wild type.*

Experiment		NADPH-nitrate reductase		Inducible NADPH-cytochrome *c* reductase		$FADH_2$-nitrate reductase		MVH-nitrate reductase	
		Enzyme Units per Assay Tube							
		Unind.	Ind.	Unind.	Ind.	Unind.	Ind.	Unind.	Ind.
Mixtures									
Induced *nit-1* +	*nit-2*	8.2	5.0	38.2	36.2	2.8	3.2	3.2	2.4
	nit-3	6.8	1.8	33.4	38.4	1.1	11.1	12.1	161.5
	Wild type	4.7	25.8	36.6	53.5	2.5	7.1	3.0	16.4
Uninduced *nit-1* +	*nit-2*	0.0	0.0	6.2	6.8	0.0	0.0	0.0	0.0
	nit-3	0.0	0.0	8.9	6.7	1.0	14.2	10.4	198.0
	Wild type	0.0	24.4	6.4	22.8	0.0	5.6	0.0	12.2
Unmixed controls									
	nit-1	0.0	0.0	3.0	29.5	0.0	0.0	0.0	0.0
	nit-2	0.0	0.0	2.9	4.1	0.0	0.0	0.0	0.0
	nit-3	0.0	0.0	6.1	2.8	0.7	13.9	9.9	225.0
	Wild type	0.0	24.8	3.6	21.4	0.0	5.4	0.0	11.1

Mixtures containing 0.05 ml of each crude extract (i.e., 0.1 ml total for each assay) were incubated for 3 hr at 4° and assayed for the various activities as described in *Materials and Methods*. Control values represent endogenous activities present in 0.05 ml of the individual crude extracts at the end of 3 hr. The protein concentration of the individual crude extracts ranged from 6 to 11 mg/ml. The protein content of the above mixtures ranged from 0.7 to 1.0 mg/tube and the controls contained from 0.30 to 0.55 mg/tube.

in extracts of induced wild type while that of induced *nit-1* plus uninduced wild type produced yields as high as 60 per cent.

Cell-free extracts of all uninduced and induced strains displayed the same sucrose density gradient profiles for constitutive NADPH-cytochrome *c* reductase[9] as illustrated by uninduced *nit-1* (Fig. 1*A*). The profile for uninduced *nit-3* (Fig. 1*B*) also possessed a second activity zone ($S_{20,w}$ = 6.8*S*) containing both $FADH_2$- and MVH-nitrate reductases implicating the two activities with a single protein complex. The inducible NADPH-cytochrome *c* reductase which has a significantly smaller sedimentation coefficient (4.5*S*) than the ubiquitous constitutive cytochrome *c* reductase was only observed in nitrate induced *nit-1* (Fig. 1*C*).

The sedimentation behavior of complemented nitrate reductase formed in mixtures of induced *nit-1* with either uninduced *nit-2* or wild type (Figs. 2*A*, *B*) was essentially the same as that of the wild-type enzyme (Fig. 2*C*). The single peak of NADPH-nitrate reductase activity ($S_{20,w}$ = 7.9*S*) included the $FADH_2$- and MVH-nitrate reductases and the inducible NADPH-cytochrome *c* reductase activity characteristically associated with the induced wild-type

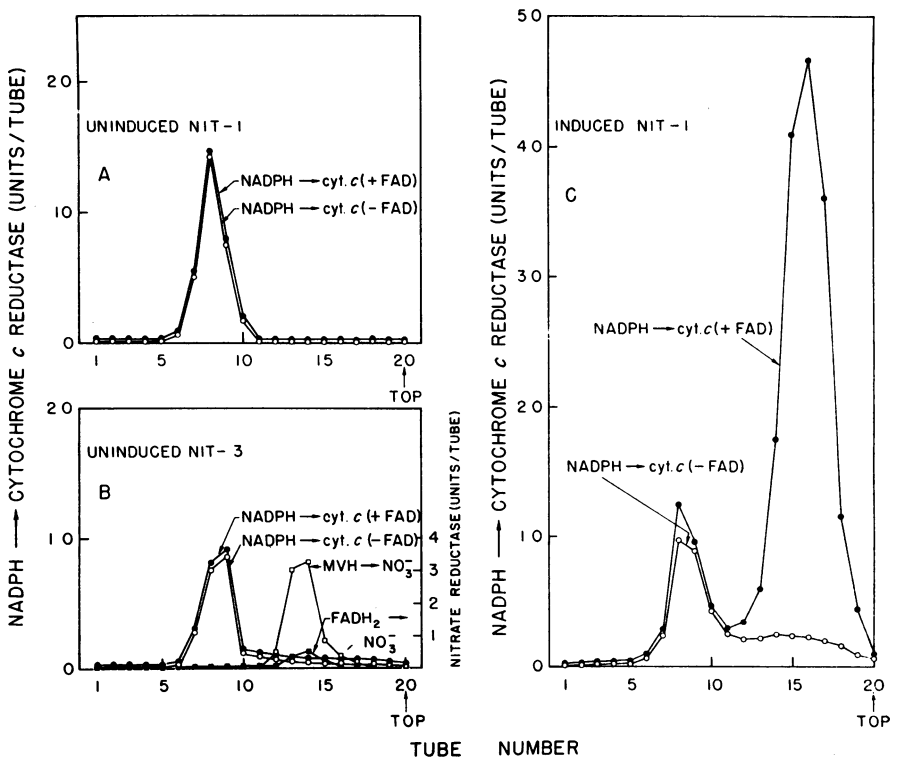


FIG. 1.—Sucrose density gradient profiles of crude extracts showing the sedimentation characteristics of the constitutive NADPH-cytochrome *c* reductase and the activities associated with the NADPH-nitrate reductase in (*A*) uninduced *nit-1*, (*B*) uninduced *nit-3*, and (*C*) induced *nit-1*. One-tenth of a ml of each crude extract was layered on linear 4.6-ml sucrose gradients (15.5–33%, w/v, in preparation buffer) and centrifuged at 39,000 rpm (SW 39L rotor) for 20 hr in a refrigerated Spinco model L ultracentrifuge. Eight-drop fractions were collected by a downward flow technique. Recoveries ranged from 30 to 50%.

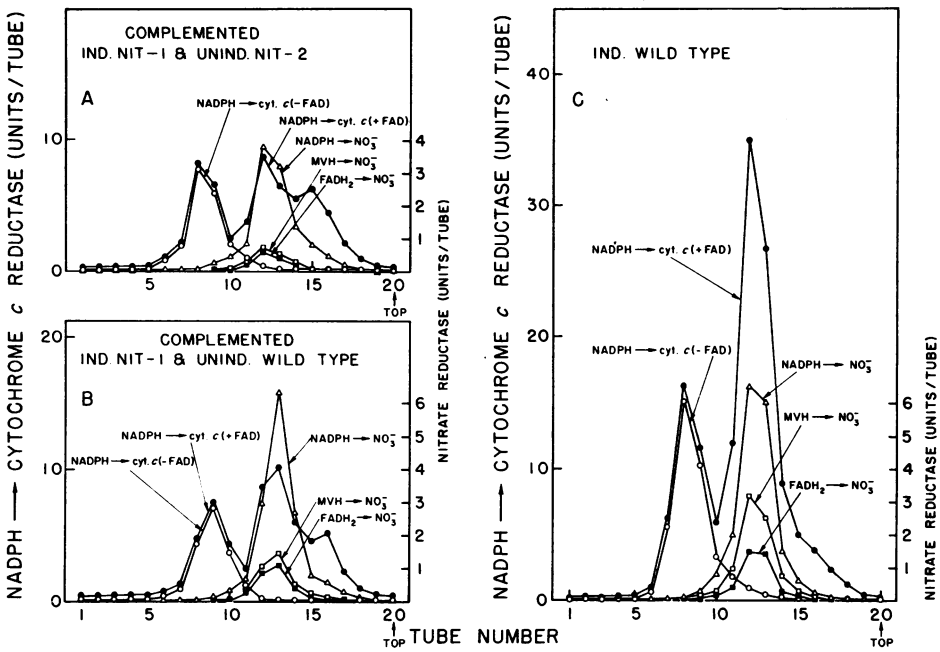


FIG. 2.—Sucrose density gradient profiles of complemented nitrate reductase and its associated activities present in crude extract mixtures of (*A*) induced *nit-1* and uninduced *nit-2* and (*B*) induced *nit-1* and uninduced wild type. Gradients (*A*) and (*B*) contain 0.1 ml of a crude extract mixture that was incubated for 10 hr at 4° prior to centrifugation. The activities associated with nitrate reductase in 0.1 ml of a crude extract from induced wild type are shown (*C*). Method of centrifugation, fractionation procedure, and recoveries are given in Fig. 1.

enzyme (Fig. 2*C*). It therefore appears that some of the slower sedimenting inducible cytochrome *c* reductase of *nit-1* (Fig. 1*C*) has shifted to a faster sedimenting form (Figs. 2*A*, *B*) identical to that of the inducible cytochrome *c* reductase associated with the wild-type nitrate reductase (Fig. 2*C*). Apparently not all of the slower sedimenting inducible cytochrome *c* reductase has been utilized in the complementation reaction since some is still evident in the sucrose density profile (Figs. 2*A*, *B*). A somewhat similar profile has been obtained with the crude extract mixture of induced *nit-1* and induced or uninduced *nit-3* (not shown). However, the pattern is complicated in other respects and will be presented in a future publication.

The formation of NADPH-nitrate reductase in a crude extract mixture of *nit-1* and uninduced wild type displayed a sharp pH optimum between 6.5 and 7.0. The complementing activities of crude extracts of induced *nit-1* and uninduced *nit-2*, *nit-3* and wild type are extremely heat-labile (losing 80 to 100 per cent of their activity after 5 minutes at 38°), inactivated by tryptic digestion, and markedly decreased by overnight storage at 4° or −15°.

After 3 hours of high-speed centrifugation the complementing activity of uninduced *nit-2*, *nit-3* and wild type occurred mainly (*ca.* 90 per cent) in the pellet, whereas that of the crude extract of *nit-1* had not sedimented (Table 2). The apparent particulate complementing activity of *nit-2*, *nit-3*, and wild type is not associated with the mitochondria isolated by the procedure of Greenawalt

TABLE 2. *Distribution of the in vitro complementation activity of Neurospora crassa wild-type and mutant strains during differential centrifugation.*

Fraction	NADPH-nitrate reductase formed during 15 min complementation* Induced *nit-1* Total units	Spec. act.	Uninduced *nit-2* Total units	Spec. act.	Uninduced *nit-3* Total units	Spec. act.	Uninduced Wild Type Total units	Spec. act.
20,000 × *g* (20′) sup.	5106	17.0	756	4.4	1920	8.7	3320	8.0
20,000 × *g* (20′) pellet	0	0.0	462	1.9	2058	11.2	5810	25.5
144,000 × *g* (1 hr) sup.	5120	30.7	194	2.0	761	4.2	1308	6.8
144,000 × *g* (1 hr) pellet	4	0.0	221	7.9	1060	25.2	1780	38.6
144,000 × *g* (3 hr) sup.†	3720	23.0	162	2.6	210	1.7	356	3.5
144,000 × *g* (3 hr) pellet†	0	0.0	66	2.4	131	7.7	284	27.8

Crude extracts (20,000 × *g* supernatant solutions) of *Neurospora crassa* were prepared as described in *Materials and Methods* using the preparation buffer containing 10% glycerol.

* The uninduced fractions of *nit-2*, *nit-3*, and wild type were assayed with FMN after 15 min of incubation (25°) with the 20,000 × *g* supernatant solution of induced *nit-1*; whereas, the induced *nit-1* fractions were assayed after an identical incubation with the 144,000 × *g* pellet (1 hr) of uninduced wild type.

† The 3-hr fractions were obtained by decantating the 144,000 × *g* (1 hr) supernatant solution and recentrifuging for an additional 2 hr.

et al.,[15] or with mitochondrial fragments using cytochrome oxidase activity as a mitochondrial marker. Despite the seemingly particulate nature of one member of each complementing pair, the NADPH-nitrate reductase produced by complementation is soluble as is the wild-type enzyme.

Both the extent and linear rate of *in vitro* complementation at 25° experienced a 20-fold increase as compared to 2° (Fig. 3). The use of the high-speed pellet of uninduced *nit-2*, *nit-3*, or wild type and a nitrite reductase-free fraction of induced *nit-1* has made possible the routine demonstration of highly reactive complementing mixtures requiring relatively short incubation periods of 10–30 minutes at 25° (Fig. 3). The formation of NADPH-nitrate reductase by *in vitro* complementation was linear for a certain range of increasing concentrations of the induced *nit-1* preparation using a constant level of uninduced *nit-2*, *nit-3*, or wild-type extract, and for increasing concentrations of uninduced *nit-2*, *nit-3*, or wild-type preparations using a constant level of the induced *nit-1* extract (not shown). FMN is more desirable than FAD in the NADPH-nitrate reductase assay since it will not substitute for the FAD requirement of

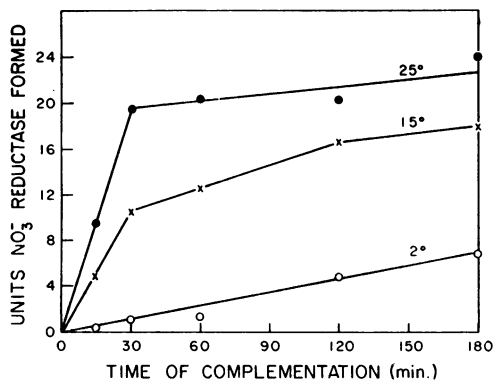


FIG. 3.—Time course of complementation at different temperatures. The ammonium sulfate fraction of *nit-1* (3.3 mg protein) and the 144,000 × *g* (1 hr) pellet of uninduced wild type (16.0 mg protein) were mixed together in a total volume of 9.0 ml of preparation buffer containing 20% glycerol. At the times indicated, 0.3 ml of the mixture was removed and assayed for NADPH-nitrate reductase for 15 min at 25° with FMN in place of FAD as described in **Materials and Methods.**

any nitrite reductase that might be present. It also produces a higher NADPH-nitrate reductase activity in the wild-type and complemented enzymes (unlike its effect on the enzyme from *N. crassa* 5297a[7]).

The partially purified NADPH-nitrate reductase formed by *in vitro* complementation of induced *nit-1* with either uninduced *nit-2*, uninduced *nit-3*, or uninduced wild type was similar in most properties to the purified wild-type enzyme[7] including molecular weight, pH optima substrate and cofactor affinities, presence of cytochrome b_{557}, sensitivity to metal binding agents and —SH inhibitors, and response to 50° treatment.[7] It differed, however, in the ratio of its associated activities (Fig. 2).

Discussion. The formation of an assimilatory NADPH-nitrate reductase similar to the inducible wild-type enzyme by *in vitro* complementation using extracts of separately grown non-allelic *Neurospora* mutants exhibited three fundamental features: (*a*) in all instances one member of the complementing pair was *nitrate-induced nit-1* which uniquely possesses inducible NADPH-cytochrome *c* reductase, (*b*) prior induction of the other mutants was unessential for complementation (uninduced wild type which resembled *nit-2* in its lack of enzymatic activities, also complemented with induced *nit-1*), and (*c*) complementation resulted in the appearance or stimulation of $FADH_2$- and MVH-nitrate reductase activities.

Although there is no direct evidence for the molecular basis of the above phenomenon several hypotheses can be considered. First, the possibility of *de novo* protein synthesis under the experimental conditions is almost certainly eliminated, particularly since preliminary experiments with high concentrations of DNase, RNase, and chloromycetin in the complementation reaction had no significant effect. The sucrose density gradient profiles (Figs. 1 and 2) showing the conversion of the slower sedimenting inducible NADPH-cytochrome *c* reductase contributed by *nit-1* to a faster-sedimenting form characteristically associated with NADPH-nitrate reductase argue against a second hypothesis that nitrate reductase formation involves activation of a pro-enzyme in one of the complementing extracts by a component of the second extract. This observation implies a combining of protein subunits as the basis for the complementation reaction, thus suggesting a third hypothesis which in our view best fits the data. More specifically, *in vitro* complementation of NADPH-nitrate reductase is postulated to result in a heteromultimer formed from the interaction of at least two protein subunits coded for by different cistrons. One of the subunits is believed to be the nitrate-inducible entity (molecular weight of *ca.* 50,000) of *nit-1*, responsible for the early part of the electron transport sequence as reflected by the inducible NADPH-cytochrome *c* reductase. The second component representing the latter part of the electron transport pathway is a constitutive subunit that is present in all uninduced strains (except *nit-1*) in an inactive form. It is activated (as indicated by the appearance of $FADH_2$- and MVH-nitrate reductase activities) by its combination with the above inducible protein subunit.

The increased $FADH_2$- and MVH-nitrate reductase activities in induced *nit-3* could be explained by the presence of an inactive inducible cytochrome *c*

reductase protein subunit (the result of the *nit-3* mutation) which has retained the capability of activating the constitutive subunit. The decreased complementing activity of induced *nit-3*, compared to that of uninduced *nit-3* (Table 1) could therefore be attributed to a decreased amount of the constitutive subunit available for complementation with added *nit-1* due to the prior attachment of the former to the altered cytochrome *c* reductase subunit.

The heteromultimer hypothesis is supported by additional observations. Acid treatment of purified wild-type enzyme causes complete loss of nitrate reductase and associated activities despite restoration of the pH to neutrality. The specific addition of partially purified complementing preparations from induced *nit-1* to the inactivated acid-treated enzyme significantly restores its nitrate reductase and associated activities. Similarly the loss of both NADPH-nitrate and -cytochrome *c* reductase activities (as well as the threefoldi ncrease in MVH-nitrate reductase) by mild heat treatment of the purified wild-type enzyme[7] can be significantly restored by addition of a complementing preparation from induced *nit-1* providing that the heat-treated enzyme is first acidified.

The source of cytochrome b_{557} (a component of the enzyme[6]) in the complementation reaction is not clear. All *Neurospora* strains, induced or uninduced, contain this component. Finally, while inducible NADPH cytochrome *c* reductase has proven to be a useful parameter of complementation activity in *nit-1* extracts, it is not a foolproof criterion. Partially purified fractions of inducible cytochrome *c* reductase have been prepared which lack complementing activity; and under certain conditions complete inhibition of the inducible cytochrome *c* reductase of the purified wild-type enzyme can be attained without affecting NADPH-nitrate reductase activity.

We are indebted to Helene Y. Cambier and James S. Plowden for their invaluable technical assistance.

* Contribution No. 579 of the McCollum-Pratt Institute. This investigation was supported in part by research grant AM-12788 from the National Institutes of Health, U.S. Public Health Service.

† Postdoctoral trainee, National Institutes of Health training grant (GM-57 and GM-504).

‡ Postdoctoral fellow, National Institutes of Health, U.S. Public Health Service.

§ *nit-25376*, *nit-2003*, *nit-2(983)*, *nit-4*, and *nit-5*. The latter two are possibly nitrite reductase and not nitrate reductase mutants.

[1] Nason, A., and H. J. Evans, *J. Biol. Chem.*, **202**, 655 (1953).

[2] Nicholas, D. J. D., A. Nason, and W. D. McElroy, *J. Biol. Chem.*, **207**, 341 (1954).

[3] Nicholas, D. J. D., and A. Nason, *J. Biol. Chem.*, **207**, 353 (1954).

[4] *Ibid.*, **211**, 183 (1954).

[5] Kinsky, S. C., and W. D. McElroy, *Arch. Biochem. Biophys.*, **73**, 466 (1958).

[6] Garrett, R. H., and A. Nason, these PROCEEDINGS, **58**, 1603 (1967).

[7] Garrett, R. H., and A. Nason, *J. Biol. Chem.*, **244**, 2870 (1969).

[8] Cove, D. J., and J. A. Pateman, *Nature*, **198**, 262 (1963).

[9] Sorger, G. J., *Biochim. Biophys. Acta*, **118**, 484 (1966).

[10] Sorger, G. J., and N. H. Giles, *Genetics*, **52**, 777 (1965).

[11] Azoulay, E., J. Puig, and P. Couchoud-Beaumont, *Biochim. Biophys. Acta*, **171**, 238 (1969).

[12] Antoine, A., and A. Nason, *Fed. Proc.*, **28**, 529 (1969).

[13] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

[14] Martin, R. G., and B. N. Ames, *J. Biol. Chem.*, **236**, 1372 (1961).

[15] Greenawalt, J. W., D. O. Hall, and O. C. Wallis, in *Methods in Enzymology*, ed. R. W. Estabrook and M. E. Pullman (New York: Academic Press, 1967), vol. 10, p. 142.