INDUCTION AND REPRESSION OF NITRATE REDUCTASE IN *NEUROSPORA CRASSA*

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Received for publication June 13, 1961

ABSTRACT

KINSKY, STEPHEN C. (Washington University, St. Louis, Mo.). Induction and repression of nitrate reductase in *Neurospora crassa*. J. Bacteriol. **82:**898–904. 1961.—Techniques are described for studying induced enzyme formation in *Neurospora crassa*. The effects of various parameters (time, pH, nitrate concentration, etc.) on the induction of nitrate reductase were investigated. It was demonstrated that NH_4^+, which is the end product of the metabolic sequence initiated by nitrate reductase, repressed formation of the enzyme. Parallel to the formation of nitrate reductase there was an increase in reduced triphosphopyridine nucleotide (TPNH)-cytochrome *c* reductase activity. The relationship of these two activities to each other and to the constitutive TPNH-cytochrome *c* reductase present in noninduced mycelia is discussed.

The formation of inducible enzymes in bacteria has been extensively investigated. Analogous kinetic studies in molds have not generally been attempted because of the relatively slow growth of these organisms and the difficulties involved in obtaining uniform and homogeneous cell populations. Similar obstacles are encountered in the study of adaptive enzyme formation in mammalian systems. Such studies are, nevertheless, desirable in order to test the general validity of conclusions drawn from investigations on bacteria.

In the present communication, characteristics of the induced formation of nitrate reductase in *Neurospora crassa* are described. The following considerations prompted examination of this particular enzyme: (i) *N. crassa* can be easily grown. (ii) Nitrate reductase can be rapidly assayed. (iii) The enzyme has been partially purified and its mechanism of action investigated (Nicholas and Nason, 1954). (iv) Several distinct mutants of *N. crassa*, lacking nitrate reductase, exist (Silver and McElroy, 1954). Recently, a method of preparing *Neurospora* protoplasts has been described, thus making feasible a comparison of induced enzyme formation in whole mycelia and protoplasts of this organism (Bachmann and Bonner, 1959).

MATERIALS AND METHODS

Chemicals. Flavin adenine dinucleotide (FAD) and chemically or enzymatically reduced triphosphopyridine nucleotide were obtained from the Sigma Chemical Company, St. Louis, Mo.

Activity determination. In the presence of FAD, nitrate reductase catalyzes the irreversible reduction of nitrate to nitrite by the reduced form of triphosphopyridine nucleotide (TPNH). Activity was consequently determined by a modification of the method based on the measurement of nitrite production. Sufficient "standard assay mixture" was prepared before each assay; the mixture contained the following components per milliliter: $NaNO_3$, 23 μmoles; TPNH, 0.92 μmoles; FAD, 0.09 μmoles; and potassium phosphate, 450 μmoles; pH 7.4. Each assay tube contained 44.2 μliters of the standard assay mixture, between 5 and 40 μliters of enzyme, depending on the activity, and distilled water to give a final volume of 100 μliters. All additions were made from Linderstrøm-Lang micropipettes. The reaction was started by the addition of enzyme and the tubes incubated at 30 C for 20 min. The reaction was stopped with 0.5 ml of acid sulfanilamide (1 g sulfanilamide in 100 ml of 25% perchloric acid), followed by 0.5 ml of naphthylethylenediamine (20 mg in 100 ml water). After allowing 20 min for maximum diazo dye color development, insoluble protein was removed by centrifugation. The optical density at 540 mμ of the supernatant solution was determined in a Zeiss spectrophotometer, and the amount of nitrite present was calculated from a standard curve. With these assay conditions, nitrite production was proportional to extract concen-

tration over an extended range. Any interference of nitrite reductase was minimized by using microquantities of extract prepared as described below. The activity of each extract was determined at two concentrations and expressed as mμmoles of nitrite produced in 20 min per ml of extract.

TPNH-cytochrome *c* reductase was determined essentially as described previously (Kinsky and McElroy, 1958). Each cuvette contained 0.06 μmole of TPNH, 1 μmole of NaCN, 0.03 μmoles of FAD, and 200 μmoles of potassium phosphate, pH 7.4. Cytochrome *c* (0.05 ml of a 2% solution), enzyme (0.01 to 0.10 ml), and distilled water were added to give a final volume of 1.0 ml. Sodium cyanide was present to inhibit any cytochrome *c* oxidase activity in the extract which would otherwise interfere with the assay. The reaction was started by the addition of extract, and the increase in optical density at 550 mμ, measured in a Zeiss spectrophotometer, was followed every 15 sec for 2 min. During this interval the rate of reduction remained constant and the amount of reduced cytochrome *c* formed was calculated, using a value of 2.10×10^4 cm^2/mole for the difference in molar extinction coefficients between reduced and oxidized cytochrome *c* (Massey, 1959). TPNH-cytochrome *c* reductase activity was expressed as mμmoles cytochrome *c* reduced in 20 min, calculated from the initial rate of reduction, per milliliter of extract. DPNH-cytochrome *c* reductase was assayed in an identical manner with the substitution of equivalent quantities of reduced diphosphopyridine nucleotide (DPNH) for TPNH.

Growth and induction. The minimal Fries medium described by Nason and Evans (1953) was employed in all experiments. In preliminary experiments on induction, this medium was supplemented by the incorporation of inducer (nitrate) and added to Erlenmeyer flasks (25 ml medium per 125-ml flask), which were subsequently inoculated with conidia of *Neurospora* strain 5297a. The mycelia were harvested at varying time intervals, dried as described below, and extracts prepared and examined for nitrate reductase activity. With this procedure, poor reproducibility was obtained, and comparison between experiments was generally unreliable. Reproducible results could, however, be obtained if the cells used for induction were derived from a single mycelial mat, which was grown as a standing culture to minimize sporulation and in the absence of nitrate. Basal growth medium (500 ml), containing NH_4Cl as the sole nitrogen source, was added to a 2.5-liter Fernback flask, inoculated with a heavy spore suspension, and incubated at 25 C for exactly 96 hr. The mycelium was then harvested in a large Büchner funnel; particular care was taken to maintain the circular shape and structural integrity of the mat. The mycelium was washed extensively with distilled water, and, while still on the filter paper, the mat was cut with scissors into 16 approximately equivalent pie-shaped pieces. Each section was then peeled from the paper and added to 25 ml of induction medium (see below) contained in 125-ml Erlenmeyer flasks. These flasks were vigorously shaken on a Gyrotory Shaker (New Brunswick Scientific Company, New Brunswick, N. J.) maintained at 25 C to induce rapid formation of the enzyme. No attempt was made to employ sterile techniques, since enzyme production under these conditions was complete after 4 hr. Mycelial sections from duplicate induction flasks were harvested by Buchner filtration at different times and dried over P_2O_5 in an evacuated desiccator for 12 hr. Control experiments showed that extracts (see below) prepared from the dried sections had activity identical to extracts prepared from quick frozen sections, and there was no reason to suspect a loss of nitrate and cytochrome *c* reductase during the drying operation.

Maximum formation of enzyme was obtained in a medium which did not contain all the components required for maximum growth. The complete induction medium contained per liter: sucrose, 20 g; KH_2PO_4, 1 g; $NaNO_3$, 2 g; and 3.2 g (0.02 moles) dimethylglutaric acid as buffer. The pH was adjusted to 4.5 with a few drops of 10 N NaOH. The effect on enzyme production of modifying this "complete induction medium" will be described below.

Extract preparation. The dried mycelial sections were ground to a fine powder with a mortar and pestle. Between 30 and 40 mg of the powder was weighed into a 15 by 95 mm Lusteroid centrifuge tube, and 3 ml of 0.05 M potassium phosphate buffer, pH 7.5, containing 10^{-3} M ethylenediaminetetraacetic acid and 10^{-3} M glutathione, was added. The mixture was homogenized for exactly 2 min in a Lourdes Multimixer (Lourdes Instru-

ment Corp., Brooklyn, N. Y.) with the tube immersed in an ice water-salt bath maintained at −5 C. After this treatment the blades of the mixer were rinsed with approximately 2 ml of distilled water and the wash combined with the homogenate. The resulting suspension was centrifuged at 20,000 × *g* for 10 min in a Servall RC-2 refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The precipitate was discarded after pouring the supernatant into a calibrated test tube to determine the final volume of extract. The results reported in Table 1 show that a single homogenization was sufficient to extract all of the nitrate and TPNH-cytochrome *c* reductase. Re-extraction by homogenization of the residue derived from the first centrifugation did not result in the appearance of additional activity in the supernatant.

Table 1 also shows that 94% of the soluble protein (determined by the procedure of Lowry et al., 1951) was extracted by a single homogenization. In other experiments only 70% of the soluble protein was extracted initially, although in every instance all of the nitrate-cytochrome *c* reductase activity was present in the supernatant. This precluded reliable determination of enzymatic activity in the mycelial sections on a protein basis, and all results reported below indicate the mμmoles of nitrite formed or cytochrome *c* reduced in 20 min per mg dry weight of mycelium ("specific activity"). In each experiment, the average of specific activities from duplicate flasks is given. Unlike investigations with bacterial systems, simultaneous determination on the same sample of increase in enzymatic activity and dry weight could not be made. However, in control experiments it was estimated that during incubation (usually 2 hr in a medium producing optimum enzyme formation) growth (based on dry weight) was less than

TABLE 1. *Extraction of reductase*

Treatment	Total units in supernatant		Total mg protein in supernatant
	Nitrate reductase	TPNH-cytochrome *c* reductase	
(1) Initial extraction	1644	7130	5.81
(2) Residue from 1 re-extracted	0	0	0.47
(3) Residue from 2 re-extracted	0	0	0

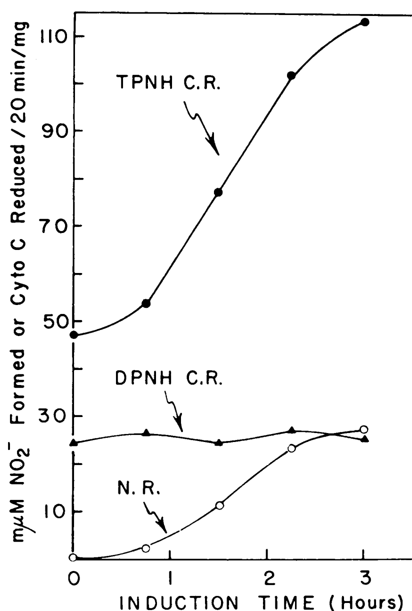


FIG. 1. *Time course of enzyme induction. Mycelial sections were incubated for various times in the complete induction medium described in the text. Sections were then harvested, dried, and extracts prepared and assayed for nitrate reductase (N.R.), TPNH-cytochrome c reductase (TPNH C.R.), and DPNH-cytochrome c reductase (DPNH C.R.).*

10% of the initial value. In all likelihood, therefore, the values cited represent a true differential rate of enzyme synthesis.

RESULTS

Nitrate reductase induction. Figure 1 shows the effect of incubation time on the induction of nitrate reductase. No enzyme was detected initially, but, after a lag phase of approximately 45 min, enzyme synthesis proceeded maximally and essentially linearly for about 2½ hr. Enzyme production decreased thereafter and, as indicated in other experiments, was completed after 4 hr.

The rate of enzyme formation was markedly dependent on the pH of the induction medium, as shown in Fig. 2. At pH values slightly above 5, there was a sharp decline in the amount of nitrate reductase formed. (During the course of this experiment the effects of various buffers were tested at pH 5.0 and 0.02 M final concentration. Succinate and dimethylglutaric acid were without effect, but citrate and acetate resulted in a complete inhibition of enzyme induction.)

The effect of increasing concentrations of

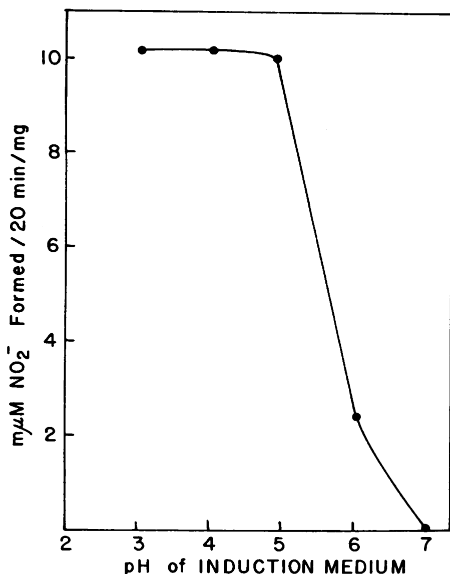


FIG. 2. *Effect of pH on rate of nitrate reductase induction. The complete induction medium, buffered with 0.02* M *dimethylglutaric acid, was adjusted to various pH values with either 10* N *NaOH or 10* N *HCl. After 2 hr incubation, mycelial sections were harvested, dried, and extracts prepared and assayed for nitrate reductase.*

$NaNO_3$ on the rate of enzyme formation is indicated in Fig. 3. Half-maximal induction was obtained at a concentration of approximately 10^{-4} M $NaNO_3$. High concentrations of $NaNO_3$ inhibit enzyme formation. At molar concentrations of 0.046, 0.091, 0.137, and 0.23, the nitrate reductase activities were, respectively, 67, 18, 4.7, and 0% of the value obtained at optimum concentrations.

Repression by ammonium chloride. The lag phase in the time course of induction may have been due to nongratuitous conditions for enzyme formation (Cohn, 1957). Thus, in the absence of any other nitrogen source, nitrate functioned not only as inducer but also provided the nitrogen required for protein synthesis. The lag phase may be indicative of the time necessary to reduce a significant amount of nitrate to the amino level. The effect of nitrate on induction was consequently tested in the presence of substrate levels of NH_4Cl. It was consistently found that, rather than shorten the lag phase, NH_4Cl depressed the levels of nitrate reductase formed (Table 2). Neither KCl nor NaCl exerted any significant effect. NH_4Cl at a final concentration of 10^{-2} M did not interfere with the standard assay. An interesting feature of the repression by NH_4Cl is indicated by the data of Table 3. Once nitrate reductase formation had begun, the subsequent addition of NH_4Cl neither stopped nor reduced enzyme synthesis. NH_4Cl must be present initially in the induction medium to demonstrate any effect.

Simultaneous induction of cytochrome c reductase activity. In an earlier investigation concerned

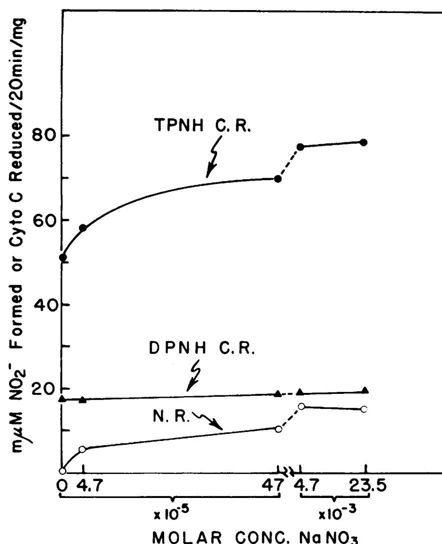


FIG. 3. *Effect of $NaNO_3$ concentration on enzyme induction. The complete induction medium was modified to contain the final concentrations of $NaNO_3$ indicated on the abscissa. After 2 hr incubation, mycelial sections were harvested, dried, and extracts prepared and assayed for nitrate reductase (N.R.), TPNH-cytochrome c reductase (TPNH C.R.), and DPNH-cytochrome c reductase (DPNH C.R.).*

TABLE 2. *Repression of nitrate reductase induction by NH_4Cl**

NH_4Cl concn ($M \times 10^3$)	Specific activity of nitrate reductase	
	Expt. 1	Expt. 2
0	13.4	17.1
1.87	10.9	12.4
18.7	7.3	6.8

* The complete induction medium was modified to contain the concentrations of NH_4Cl cited. After 2 hr incubation, mycelial sections were harvested, dried, and extracts prepared and assayed for nitrate reductase.

TABLE 3. *Effect of NH_4Cl after induction of nitrate reductase**

Incubation time	Specific activity of nitrate reductase		
	No NH_4Cl added	NH_4Cl added at 1.5 hr	NHCl added initially
hr			
0	0	—	—
0.75	5.88	—	—
1.50	19.2	—	—
2.25	27.5	27.8	—
3.00	32.5	31.5	19.1

* Experimental conditions as described in the legend to Fig. 1. To one set of flasks, NH_4Cl (final concentration: 1.87×10^{-2} M) was added at the start of the experiment; to another set, it was added after 1.5 hr incubation.

with the substrate specificity of nitrate reductase, a close parallel during enzyme purification between nitrate and TPNH-cytochrome *c* reductase activity was noted (Kinsky and McElroy, 1958). These results, and the identical Michaelis constants for both TPNH and FAD, suggested that the two enzymatic activities were shared by a common protein. This relationship was examined further in the present study.

Figures 1 and 3 show that conditions which promoted nitrate reductase formation also resulted in the induction of TPNH-cytochrome *c* reductase activity. This was demonstrated when enzyme appearance was determined as a function of induction time (Fig. 1) or nitrate concentration (Fig. 3). Since nitrate reductase is highly specific for TPNH as electron donor (Nason and Evans, 1953), little effect on DPNH-cytochrome *c* reductase should be observed if nitrate and TPNH-cytochrome *c* reductase activities are catalyzed by the same enzyme. No change in DPNH-cytochrome *c* reductase levels was seen during induction.

Figures 1 and 3 also show that there is a significant amount of TPNH-cytochrome *c* reductase present before induction or when nitrate was omitted from the induction medium. The parallel increase in nitrate reductase and TPNH-cytochrome *c* reductase is more apparent if this initial value, representing "constitutive" activity, is subtracted from the activity formed during the course of induction (or in the presence of increasing concentrations of $NaNO_3$), and the results expressed as percentages of maximum activity induced (Table 4).

The suggestion that nitrate and TPNH-cytochrome *c* reductase activities are catalyzed by a common protein was further supported by determining the effect of energy source, oxygen,

TABLE 4. *Parallel induction of nitrate and TPNH-cytochrome c reductase**

Variable	Maximal induction of	
	Nitrate reductase	TPNH-cytochrome c reductase
	%	%
Incubation time (hr)		
0	0	0
0.75	8	10
1.50	41	45
2.25	87	83
3.00	100	100
Nitrate concn (M)		
0	0	0
4.7×10^{-5}	35	26
4.7×10^{-4}	66	68
4.7×10^{-3}	100	97
2.35×10^{-2}	97	100

* The data of Fig. 1 and 3 have been recalculated to show the percentage of maximum activity which was induced after subtracting, from the TPNH-cytochrome c reductase values, the constitutive activity present under conditions of no induction.

TABLE 5. *Effect of sucrose, O_2, and NH_4Cl on induction of nitrate and TPNH-cytochrome c reductase**

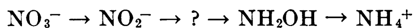
Modification of induction medium	Maximal induction of	
	Nitrate reductase	TPNH-cytochrome c reductase
	%	%
None	100	100
NH_4Cl added (1.87×10^{-2} M)	59	41
Sucrose omitted	37	24
O_2 omitted (evacuated)	36	38

* The complete induction medium was modified as indicated. After 2 hr incubation, mycelial sections were harvested, dried, and extracts prepared and assayed for nitrate and TPNH-cytochrome *c* reductase. The TPNH-cytochrome *c* reductase activity was corrected for constitutive activity before calculating the percentage of maximum activity which was induced (see text).

and repressor on enzyme formation. Omission of either sucrose or O_2, or addition of NH_4Cl, resulted in a significant and nearly equivalent decrease in the amount of nitrate and TPNH-cytochrome *c* reductase activities which were induced (Table 5).

DISCUSSION

The assimilation of nitrate by *N. crassa* proceeds according to the following pathway (McElroy and Spencer, 1955):



$$NO_3^- \rightarrow NO_2^- \rightarrow ? \rightarrow NH_2OH \rightarrow NH_4^+$$

The present investigation has been concerned with the various parameters influencing the formation of nitrate reductase, which catalyzes the initial reductive step in this metabolic sequence. It has been shown that the end product of this pathway, NH_4^+, decreases the formation of nitrate reductase. This example of "feedback repression" is analogous to results observed in similar studies on various metabolic pathways in bacteria (Vogel, 1957; Gorini and Maas, 1957; Yates and Pardee, 1957; Levin and Magasanik, 1961), and presumably functions as a means of physiological control for nitrate utilization in *Neurospora*. It should be noted that "feedback inhibition" (that is, inhibition by the end product, NH_4^+) of nitrate reductase activity was not observed with extracts under the conditions of enzymatic assay. Nitric oxide has recently been suggested as the intermediate preceding hydroxylamine in this metabolic sequence (Fewson and Nicholas, 1960). The effect of these intermediates on the induced formation of nitrate reductase has not been established. Nutritional studies have demonstrated, however, that the immediate product of nitrate reductase activity, nitrite, can function as an inducer of this enzyme (Nason and Evans, 1953).

This investigation has also confirmed the feasibility of studying induced enzyme formation in *Neurospora*. Although the rate of nitrate reductase synthesis is slower than the extensively studied bacterial systems (such as β-galactosidase and penicillinase), this may constitute an inherent advantage, since it permits an examination of the early stages involved in induced enzyme formation and repression. Nitrate reductase is particularly suitable for such a study because it is the initial enzyme of a metabolic pathway for which numerous mutants of *N. crassa* are known. There are now also existent several independent nonallelic mutants which effect the synthesis of nitrate reductase (Kinsky, unpublished observations), and an extensive investigation of the genetic factors involved can be pursued.

The relationship, if any, between the constitutive TPNH-cytochrome *c* reductase (present when *Neurospora* was grown on NH_4Cl as the sole nitrogen source) and the nitrate-cytochrome *c* reductase (induced when $NaNO_3$ is added to the medium) remains unclear. The intriguing possibility exists that these two enzymes, which possess a common catalytic activity, have also a common biosynthetic route (for example, a shared ribonucleic acid template), and that the addition of inducer in some manner controls modification of the catalytically active site to permit the enzyme protein to function more efficiently as a nitrate reductase.

ACKNOWLEDGMENT

This investigation was supported by Grant No. RG-6941 from the U. S. Public Health Service.

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