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THE REGULATION OF NITRATE REDUCTASE AND CATALASE BY AMINO ACIDS IN *NEUROSPORA CRASSA*

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SUMMARY

The induction of nitrate reductase (NADPH:nitrate oxidoreductase, EC 1.6.6.3) by nitrate in *Neurospora crassa* and its control by amino acids have been studied. The growth-inhibitory amino acids, isoleucine and cysteine as well as the growth-promotory ones, glutamine, asparagine, arginine, histidine and NH_4^+, repress nitrate reductase effectively. Methionine, tryptophan, proline, aspartic acid and glutamic acid exert little control on nitrate reductase. The repression of nitrate reductase by cysteine, isoleucine, glutamine and asparagine is accompanied by inactivation of the enzyme present initially. The nitrate-induced NADPH–cytochrome *c* reductase (NADPH:cytochrome *c* oxidoreductase, EC 1.6.2.3) is also repressed by amino acids which control nitrate reductase, providing further evidence to show that these two enzyme activities may reside in the same protein.

Catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) has been found to be induced subsequent to the induction of nitrate reductase by nitrate in *N. crassa*. The induction of catalase is probably by its substrate H_2O_2 which would be formed by the interaction of the flavine component of nitrate reductase with oxygen. The amino acids which control nitrate reductase, repress catalase also. The catalase level appears to be determined by the nitrate reductase activity of the mycelia.

INTRODUCTION

Nitrate reductase (NADPH:nitrate oxidoreductase, EC 1.6.6.3) in *Neurospora crassa* has been shown to be a metalloflavoprotein containing molybdenum and FAD (refs. 1–3). This important enzyme in the nitrate reduction sequence is subject to control by various factors. Molybdenum is essential for the synthesis of this enzyme[2]. The enzyme activity is absent in those cells grown in a medium lacking nitrate[1,2]. KINSKY[4] has observed the repression of the Neurospora nitrate reductase by NH_4^+. NH_4^+ has also been found to repress nitrate reductase in *Aspergillus nidulans*[5] and *Chlorella vulgaris*[6]. SORGER[7] has found the Neurospora enzyme to be repressed by β-methylaspartate and 2-methylalanine. This enzyme has also been

shown to be repressed by the ferric trihydroxamate, XFe, elaborated by this organism[8]. Together with nitrate reductase, NADPH-cytochrome *c* reductase (NADPH: cytochrome *c* oxidoreductase, EC 1.6.2.3) is also induced by nitrate in this organism[4,7,9]. The ratio of the two enzyme activities is constant at various stages of enzyme purification[9], and it has been suggested that both the activities may be present in the same enzyme.

MORRIS AND SYRETT[6] have found that NH_4^+ does not inhibit nitrate reduction by *Chlorella vulgaris* cells which are unable to assimilate NH_4^+ due to lack of a carbon source. They conclude that NH_4^+ would have been able to inhibit only if it were assimilated. Amino acids which are sources of both nitrogen and carbon, may be expected to exert a profound effect on the enzyme level in such systems.

NICHOLAS[10] has found catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) and peroxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7) levels to be very low in molybdenum-deficient felts of *N. crassa*. He has suggested that the effect of molybdenum deficiency on the two iron-containing enzymes is indirect, resulting from a decrease in the activity of molybdenoflavoproteins, which produce H_2O_2, the common substrate for catalase and peroxidase. Catalase activity has been found to be high in *N. crassa* grown in sole nitrate medium and low in the same organism grown in sole ammonium medium[8]. The nitrate reductase activity is high in the former case and is absent in the latter. Thus there is a direct correlation between nitrate reductase and catalase activities and it appears that nitrate reductase determines the level of H_2O_2 which would in turn specify the amount of catalase to be synthesized.

In the present communication, the effect of amino acids on the induction of nitrate reductase, NADPH-cytochrome *c* reductase and catalase in *N. crassa*, is reported.

MATERIALS AND METHODS

Strain

A wild strain of *N. crassa* Em 5297 a was used.

Media

The maintenance of the organism and culture conditions were the same as described by SIVARAMA SASTRY *et al.*[11]. Briefly, the organism was grown in 10 ml basal medium in 50-ml pyrex conical flasks at pH 4.8 in stationary cultures at 30°. Two types of basal media were used depending on the nitrogen source provided. Both the media contained in g/l: glucose, 20; KH_2PO_4, 3; $MgSO_4 \cdot 7\ H_2O$, 0.5; NaCl, 0.1; $CaCl_2$, 0.1 and in μg/l: zinc, 200; manganese, 200; copper, 80; iron, 20; molybdenum, 20; and biotin, 5. In addition, the sole ammonium medium contained ammonium tartrate 2 g/l and the sole nitrate medium contained sodium nitrate 2 g/l and sodium tartrate 1 g/l.

Chemicals

FAD and NADPH were obtained from Sigma Chemical Co., St. Louis, U.S.A. Cycloheximide was obtained as a gift from Koch, Light and Co., Great Britain.

Induction and repression of nitrate reductase and catalase

N. crassa was grown in sole ammonium medium for 40 h in stationary cultures. There was uniform growth of the mycelia in all the flasks. At the exponential phase of growth, each mycelium was washed, pressed gently between folds of filter paper and transferred to 10 ml of sole nitrate medium in 50-ml conical flasks. The flasks were then shaken at room temperature in a reciprocatory shaker (stroke 10.8 cm; 84 cycles/min). After 1 h, the addition of the following compounds, each to a set of flasks, was made at a final concentration of $5 \cdot 10^{-3}$ M: ammonium tartrate, L-cysteine, DL-isoleucine, L-glutamine, L-asparagine, L-arginine, L-histidine, glycine, L-alanine, D-alanine, L-serine, L-threonine, L-lysine, L-ornithine, L-leucine, L-valine, L-phenylalanine, L-tyrosine, L-tryptophan, L-methionine, L-proline, L-glutamic acid and L-aspartic acid. There were also control flasks to which no additions were made. The shaking was then continued. The mycelia were taken out at various time intervals, washed with ice-cold water and pressed gently between folds of filter paper. Two mycelia in each case were ground with 3 ml of cold phosphate buffer (0.05 M; pH 7.0) and pyrex glass powder in a chilled glass mortar. The homogenate was centrifuged in the cold at 3000 $\times$ *g* for 15 min and the supernatant was collected and used for enzyme assays. The enzyme extracts contained 2.5–3.0 mg protein per ml as assayed by the method of LOWRY *et al.*[12].

Induction of catalase directly by H_2O_2

N. crassa mycelia grown in sole ammonium medium for 40 h were transferred to fresh sole ammonium medium containing H_2O_2 at a concentration of $1 \cdot 10^{-3}$ M in 50-ml flasks. There were also control flasks which did not contain H_2O_2. The flasks were then shaken at room temperature. After 1 h, the amino acids, L-glutamine, L-asparagine, L-alanine, L-serine, L-glutamic acid, L-leucine, L-phenylalanine, L-methionine and L-tryptophan were added, each to a set of flasks, at a final concentration of $5 \cdot 10^{-3}$ M. The shaking was then continued, the mycelia were taken out after 6 h and the induced catalase activity was assayed.

The incorporation of [2-^{14}C]glycine into the mycelial heme fraction

This study was carried out to see whether heme was synthesized during the induction of catalase in *N. crassa*. The mycelia, grown in sole ammonium medium for 40 h, were transferred, to sole nitrate and sole ammonium media, both containing [2-^{14}C]glycine. The flasks were then shaken at room temperature for 5 h after which the mycelia were collected and washed free of adhering radioactivity. Hemin was isolated from the mycelial acetone-dried powder according to the method of LABBE AND NISHIDA[13] after the addition of carrier hemin. The radioactivity of a known amount of isolated and recrystallized hemin was taken as a measure of the radioactivity incorporated into the mycelial heme fraction. Radioactivity measurements were made in an end-window counter. Appropriate corrections for self-absorption and background were made.

Assay of nitrate reductase

Nitrate reductase was assayed essentially according to the method of NICHOLAS, NASON AND MCELROY[2] by the estimation of the nitrite formed, with the following modifications. The reaction was allowed to proceed for 15 min and after stopping

the reaction with the acid sulfanilamide reagent the reaction mixture was diluted to 3.5 ml and then the color was developed and read. The amount of the enzyme used was carefully chosen so that not more than 40 mμmoles of nitrite were formed in any one assay. The enzyme activity was expressed as mμmoles of nitrite formed in 15 min per ml of the enzyme extract.

Assay of NADPH-cytochrome c reductase

NADPH-cytochrome *c* reductase was assayed according to the method of KINSKY[4]. The enzyme activity was expressed as mμmoles of cytochrome *c* reduced in 15 min, calculated from the initial rate of reduction per ml of the enzyme extract. The difference in the extinction coefficients of reduced and oxidized cytochrome *c* at 550 mμ was taken as 2.10×10^4 cm^2/mmole (ref. 14).

Assay of catalase

Catalase was assayed by the permanganate titration method of RAMACHANDRAN AND SARMA[15]. The catalase activity was expressed as μmoles of H_2O_2 decomposed in 5 min per ml of the enzyme extract.

Assay of nitrate in the medium

The nitrate content of the medium was assayed essentially according to the method of NICHOLAS AND NASON[16]. The culture filtrate from each flask was freed of chloride by Ag_2SO_4 treatment and made up to 20 ml. Out of this, 0.5-ml aliquots were pipetted out into 50-ml conical flasks and 1 ml of the phenol disulfonic acid reagent was added. After 10 min 10 ml of water and 10 ml of the ammonia solution were added, the reaction mixture was made up to 25 ml and the color was read at 420 mμ in a Klett–Summerson colorimeter.

RESULTS

Induction of nitrate reductase and NADPH-cytochrome c reductase by nitrate

The time course of the induction of nitrate reductase and NADPH-cytochrome *c* reductase is given in Fig. 1. Before the transfer to sole nitrate medium the mycelia did not have any nitrate reductase activity, but had considerable 'constitutive' NADPH-cytochrome *c* reductase activity. Nitrate reductase was induced with a lag period of about 30 min. For another 2 h the enzyme activity increased exponentially with time. After 3 h there was no further increase in nitrate reductase activity. NADPH-cytochrome *c* reductase activity increased linearly with time and maximum activity was reached after 3 h. Cycloheximide was found to inhibit nitrate reductase synthesis completely at a concentration of 5 μg/10 ml (Table I) thereby indicating *de novo* protein synthesis.

Induction of catalase by nitrate

Fig. 2 gives the time course of the induction of catalase by nitrate in *N. crassa*. The catalase activity of the mycelia before the transfer to sole nitrate medium was low. Maximum catalase activity was reached 7 h after the transfer and there was no further increase in enzyme activity. While nitrate reductase reached maximum activity 3 h after transfer, most of the induced catalase was synthesized between

TABLE I

EFFECT OF CYCLOHEXIMIDE ON THE INDUCTION OF NITRATE REDUCTASE IN *N. crassa*

N. crassa mycelia grown for 40 h in sole ammonium medium were transferred to sole nitrate medium. Glutamine at a final concentration of $5 \cdot 10^{-3}$ M was added 1 h after the transfer. Cycloheximide was added at a final concentration of 5 μg/10 ml under different conditions as indicated below.

Addition to the induction medium	*Time after transfer at which nitrate reductase was assayed (h)*	*Nitrate reductase (units*/ml extract)*
Nil	1	171
Cycloheximide added at the time of transfer	1	0
Nil	3	409
Cycloheximide added at the time of transfer	3	0
Cycloheximide added 1 h after transfer	3	171
Glutamine added 1 h after transfer	3	43
Glutamine + cycloheximide added 1 h after transfer	3	6

* One unit of nitrate reductase is the amount of the enzyme catalyzing the formation of 1 mμmole of nitrite in 15 min.

the 4th and 8th h of shaking in sole nitrate medium (Fig. 2). This situation could be interpreted as follows. After the maximum activity of nitrate reductase is reached, the formation of H_2O_2 in substantial amounts would begin as a result of the interaction of the flavin component of nitrate reductase with oxygen. The excess of H_2O_2 formed must be disposed of by the cells and hence more catalase would be induced.

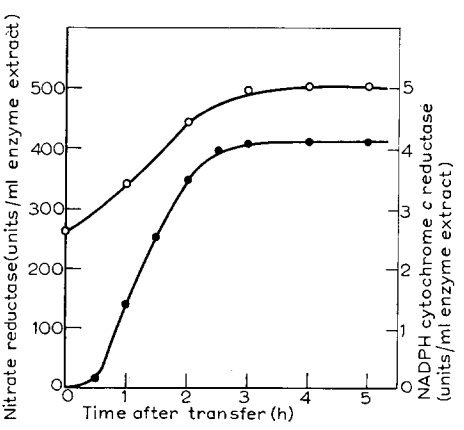

Fig. 1. Induction of nitrate reductase and NADPH-cytochrome *c* reductase. For experimental details see text. ●—●, nitrate reductase activity; ○—○, NADPH-cytochrome *c* reductase activity. One unit of nitrate reductase is the amount of enzyme catalyzing the formation of 1 mμmole of nitrite in 15 min. One unit of NADPH-cytochrome *c* reductase is the amount of enzyme catalyzing the formation of 1 μmole of reduced cytochrome *c* in 15 min.

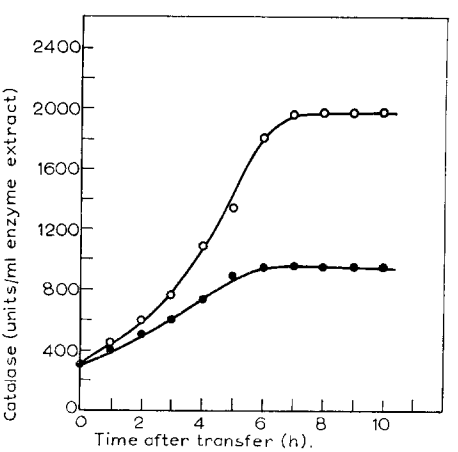

Fig. 2. Induction of catalase in *N. crassa*. For experimental details see text. ●—●, catalase activity induced after transfer to fresh ammonium medium; ○—○, catalase activity induced after transfer to sole nitrate medium. One unit of catalase is the amount of enzyme catalyzing the decomposition of 1 μmole of H_2O_2 in 5 min.

Cycloheximide was also found to inhibit completely the nitrate-induced synthesis of catalase indicating *de novo* protein synthesis.

Induction of catalase after transfer to sole ammonium medium

Fig. 2 also gives the extent of catalase induction following the transfer of sole ammonium-grown mycelia to fresh sole ammonium medium. In this case also catalase synthesis was observed but this was not at all comparable to the amount of catalase induced by nitrate. The former is perhaps mainly due to the increased aeration that the mycelia were subjected to during the process of shaking.

TABLE II

SYNTHESIS OF HEME DURING THE INDUCTION OF CATALASE IN *N. crassa*

N. crassa mycelia grown for 40 h in sole ammonium medium were transferred to sole nitrate and fresh sole ammonium media containing [2-^{14}C]glycine at a level of 240 000 counts/min per 10 ml medium and shaken. After 5 h the extent of the incorporation of the label into the mycelial heme fraction in each case was determined.

Induction medium	*Total mycelial uptake (counts/min per 100 mg dry wt.)*	*Incorporation into heme (counts/min per mg hemin per 100 mg dry wt.)*	*Total uptake (%)*
Nitrate	271 031	58 531	21.60
Ammonium	242 656	20 778	8.56

Heme synthesis during catalase induction

Table II gives data on the incorporation of [2-^{14}C]glycine into the mycelial heme fraction. The results indicate the synthesis of heme during catalase induction following transfer to both types of media. But the incorporation into heme fraction of the mycelia transferred to sole nitrate medium was more than twice that into the heme fraction of the mycelia transferred to sole ammonium medium. Thus, heme synthesis and overall catalase induction were found to follow the same pattern.

Repression of nitrate reductase and catalase by amino acids

The effect of the products of nitrate reduction on the induction of nitrate reductase and catalase is listed in Table III. Nitrite at non-lethal concentrations ($1 \cdot 10^{-3}$ M) did not repress nitrate reductase. In fact nitrite has been found to be an inducer of nitrate reductase in *N. crassa*[9]. Two other reduction products of nitrate, hyponitrite, because of its instability, and hydroxylamine, because of its toxicity, could not be tried in this system. NH_4^+, added as ammonium tartrate at a concentration of $5 \cdot 10^{-3}$ M was found to repress nitrate reductase in *N. crassa*. KINSKY[4] found NH_4^+ to repress nitrate reductase only when included before enzyme induction. He observed that after enzyme formation had begun, the subsequent addition of NH_4Cl neither stopped nor reduced enzyme synthesis. On the contrary, in the present studies, ammonium tartrate was found to repress nitrate reductase even when added 1 h after the transfer to sole nitrate medium. Sodium tartrate was not found to have any effect.

Many amino acids at a concentration of $5 \cdot 10^{-3}$ M repress the synthesis of

TABLE III

REPRESSION OF NITRATE REDUCTASE AND CATALASE BY AMINO ACIDS IN *Neurospora crassa*

The additions of amino acids at a final concentration of $5 \cdot 10^{-3}$ M were made 1 h after the transfer of mycelia to sole nitrate medium. Before the transfer the catalase activity of the mycelia was 302 units/ml enzyme extract. At the time of the additions the nitrate reductase activity was 173 units, and the catalase activity 446 units/ml enzyme extract. The mycelial dry weight was 21 mg before the transfer and 23 mg at the time of the additions. Mycelia were taken out at time intervals after the additions and the total nitrate reductase and catalase activities determined. The specific activities of the enzymes were found to follow the same pattern as their total activity. One unit of nitrate reductase is the amount of enzyme catalyzing the formation of 1 mμmole of nitrite in 15 min. One unit of catalase is the amount of enzyme catalyzing the decomposition of 1 μmole of H_2O_2 in 5 min. The enzyme extract contained 2.0–2.5 mg protein/ml.

Group No.	*Additions to the induction medium*	*Nitrate reductase (units/ml enzyme extract) Time after addition*		*Catalase (units/ml enzyme extract) Time after addition*		*Mycelial dry wt. (mg) Time after addition*		
		2 h	*4 h*	*4 h*	*6 h*	*2 h*	*4 h*	*6 h*
	None	409	409	1062	1875	27.0	31.0	34.0
	Sodium nitrite	423	435	—	—	24.0	26.0	—
I	L-Cysteine	Nil	Nil	640	1150	23.0	24.5	25.0
	DL-Isoleucine	Nil	Nil	720	1195	23.0	25.0	25.5
II	L-Glutamine	50	8	847	1320	28.5	34.0	36.0
	L-Asparagine	56	11	850	1375	28.5	34.5	37.0
	L-Arginine	106	50	956	1675	28.0	32.5	35.5
III	L-Histidine	115	153	871	1300	27.5	32.0	35.0
	Ammonium tartrate	122	192	850	1275	28.0	32.5	36.0
IV	Glycine	91	215	940	1525	25.0	31.0	34.0
	L-Alanine	102	243	879	1535	25.5	31.5	34.0
	D-Alanine	104	251	891	1582	25.5	30.5	34.0
	L-Serine	96	243	957	1510	25.5	31.5	33.5
	L-Threonine	107	257	902	1482	26.0	32.0	34.0
	L-Lysine	97	216	1025	1960	25.0	30.5	33.5
	L-Ornithine	104	215	1042	1985	25.5	31.0	33.5
V	L-Leucine	215	286	892	1310	26.0	31.0	34.0
	L-Valine	208	284	910	1475	26.0	30.5	34.5
	L-Phenylalanine	195	280	900	1475	26.5	31.0	34.0
	L-Tyrosine	214	301	905	1450	26.5	31.0	34.0
VI	L-Methionine	280	326	933	1320	27.0	31.5	33.5
	L-Tryptophan	312	333	750	1309	30.0	37.0	40.0
	L-Aspartic acid	343	368	1190	1910	27.0	31.5	34.0
	L-Glutamic acid	347	372	1200	1950	27.0	31.0	34.0
	L-Proline	375	400	1050	1890	27.5	32.5	34.0

nitrate reductase (Table III). This would be expected to lead to decreased formation of H_2O_2 and consequent decrease in the nitrate-induced synthesis of catalase. The control on catalase exerted by these compounds would thus be indirect, their action on nitrate reductase being the primary event. In fact, this was found to be the case in our present studies. On the basis of their effect on the induction of nitrate reductase and catalase, the amino acids could be broadly divided into six groups (Table III). The addition of cysteine and isoleucine not only stopped nitrate reductase synthesis, but also totally abolished the enzyme activity already present. These amino acids were also found to repress catalase most effectively. It may be noted that these amino acids inhibited the growth of the organism. Cysteine is known to be toxic to Neurospora even at a much lower concentration[17]. It has been reported to inhibit

the cystathionase reaction in *N. crassa*[18]. Isoleucine has been found to inhibit the growth of cultured tobacco cells[19].

Glutamine, asparagine and arginine completely stopped any further synthesis of nitrate reductase. Glutamine and asparagine caused an almost complete inactivation of the nitrate reductase present before their addition, while arginine was more sparing in its action. Glutamine and asparagine were found to repress catalase effectively also, while arginine exerted only a moderate control on catalase. These amino acids were found to have a slight growth-promotory effect.

Histidine and ammonium tartrate exerted an effective control on both nitrate reductase and catalase. These compounds were also growth-promotory.

Glycine, both the enatiomorphs of alanine, serine, threonine, lysine and ornithine initially stopped nitrate reductase synthesis completely but allowed substantial quantities of the enzyme to be synthesized later on. These amino acids were found to cause only a moderate repression of catalase, with the exception of lysine and ornithine which had no effect whatsoever on catalase induction. These amino acids caused growth inhibition initially, though it was not observed at later stages.

Leucine, valine, phenylalanine and tyrosine resembled the previous group of amino acids in their effect on nitrate reductase induction, except that they allowed much more of the enzyme to be induced. Valine, phenylalanine and tyrosine caused only a moderate repression of catalase in contrast with leucine which repressed catalase effectively.

Glutamic acid, aspartic acid, proline, methionine and tryptophan had little effect on the induced synthesis of nitrate reductase. The former three amino acids had no effect on the induction of catalase also, but the latter two exerted an effective

TABLE IV

EFFECT OF SOME AMINO ACIDS ON THE INDUCTION OF CATALASE BY H_2O_2 IN *N. crassa*

N. crassa mycelia grown for 40 h in sole ammonium medium were transferred to fresh sole ammonium medium containing 10^{-3} M H_2O_2 and shaken. The addition of the amino acids at a concentration of $5 \cdot 10^{-3}$ M was made 1 h after transfer and then the shaking was continued. 6 h later, shaking was stopped and the catalase activity of the mycelia determined. Before the transfer the catalase activity was 308 units/ml extract. 7 h after transfer to sole ammonium medium lacking H_2O_2 the catalase activity was 858 units/ml enzyme extract.

Additions to the ammonium + H_2O_2 medium	*Catalase activity (units*/ml enzyme extract)*
Nil	929
L-Glutamine	931
L-Asparagine	938
L-Alanine	921
L-Serine	938
L-Glutamic acid	1487
L-Phenylalanine	901
L-Leucine	723
L-Methionine	741
L-Tryptophan	802

* One unit of catalase is the amount of the enzyme catalyzing the decomposition of 1 μmole of H_2O_2 in 5 min.

control on catalase. In addition, L-tryptophan had a high growth-promotory effect. The other amino acids had no effect on growth.

Effect of amino acids on the direct induction of catalase by H_2O_2

It was reported earlier in this communication that some amino acids behaved unexpectedly in their effect on catalase induction by nitrate. This might be due to the exertion of direct control on catalase by these amino acids unlike the other amino acids which act on catalase indirectly through their control of nitrate reductase. Therefore it was decided to examine the effect of these amino acids on catalase synthesis in a system in which nitrate reductase is absent. It can be seen from Fig. 2 that catalase synthesis could also be induced though to a much lower extent in mycelia after transfer to fresh sole ammonium medium. Inclusion of H_2O_2 at a concentration of $1 \cdot 10^{-3}$ M in this system augmented this induction (Table IV). In view of the instability and high reactivity of H_2O_2, this increase in catalase synthesis is significant since induction would take place only after an effective concentration of H_2O_2 reached the induction site. The addition of leucine, methionine and tryptophan to this system was in fact found to result in repression of catalase (Table IV). This would show that these amino acids exerted their control directly on catalase. Other amino acids like glutamine, asparagine, alanine and serine when added to this system did not repress catalase. This would be added evidence for their action on catalase effected through their control of nitrate reductase after the transfer of the mycelia to sole nitrate medium. Glutamic acid, when included in the sole ammonium medium, actually boosted the induced synthesis of catalase.

TABLE V

EFFECT OF AMINO ACIDS ON NITRATE REDUCTASE AND CATALASE ACTIVITIES *in vitro*

The enzymes were preincubated for 15 min with the following amino acids present at a concentration of $1.5 \cdot 10^{-2}$ M and then the enzyme activity was assayed.

Additions	*Nitrate reductase (units*/ml extract)*	*Catalase (units**/ml enzyme extract)*
Nil	413	1875
L-Glutamine	424	1800
Glycine	492	1825
Ammonium tartrate	431	1890
L-Isoleucine	465	1875
L-Lysine	428	1900

* One unit of nitrate reductase is the amount of enzyme catalyzing the formation of 1 mμmole of nitrite in 15 min.

** One unit of catalase is the amount of the enzyme catalyzing the decomposition of 1 μmole of H_2O_2 in 5 min.

Failure of amino acids to inhibit nitrate reductase and catalase activity in vitro

The data in Table V show the effect of ammonium tartrate and some amino acids on the activity of nitrate reductase and catalase *in vitro*, at a concentration of $1 \cdot 10^{-2}$ M. Neither ammonium tartrate nor the amino acids were found to inhibit the activity of nitrate reductase and catalase. On the other hand, these compounds acted by stimulating the activity of nitrate reductase.

TABLE VI

DETERMINATION OF THE NITRATE UPTAKE, BY THE *N. crassa* MYCELIA IN THE PRESENCE OF 'REPRESSORS'

N. crassa mycelia grown in sole ammonium medium for 40 h were transferred to sole nitrate medium. The additions of 'repressors' were made 1 h after the transfer at a final concentration of $5 \cdot 10^{-3}$ M. The nitrate uptake by the mycelia was assayed at the conditions indicated.

Time after transfer (h)	*Additions to the induction medium*	*µg nitrate nitrogen/ 10 ml medium*	*µg nitrate nitrogen taken up by a single mycelium*
0	Nil	74.8	Nil
1	Nil	69.7	5.1
3	Nil	63.5	11.3
3	L-Glutamine	58.5	16.3
3	NH_4^+	61.0	13.8
3	L-Alanine	63.0	11.8

Effect of amino acids on the uptake of nitrate

The results presented in Table VI show the effect of ammonium tartrate and some representative amino acids on the uptake of nitrate by *N. crassa* mycelia. None of these compounds was found to prevent the uptake of nitrate by the mycelia. This shows that these compounds should act at some step following the uptake of nitrate by the mycelia.

Induction and repression of NADPH-cytochrome c reductase

The effect of ammonium tartrate and some representative amino acids on the

TABLE VII

INDUCTION AND REPRESSION OF NADPH-CYTOCHROME *c* REDUCTASE IN *N. crassa*

Mycelia grown in sole ammonium medium were transferred to sole nitrate medium. The additions of amino acids were made 1 h after the transfer at a final concentration of $5 \cdot 10^{-3}$ M. Before transfer there was a constitutive enzyme activity of 2.57 units/ml extract and at the time of addition of amino acids the activity was 3.36 units. The enzyme activity was then assayed 2 h after the addition of the metabolites.

Additions to the induction medium	*NADPH-cytochrome c reductase (units*/ml extract)*
Nil	4.93
L-Glutamine	3.07
L-Alanine	3.35
Ammonium tartrate	3.64
Glycine	3.24
L-Lysine	3.18
L-Isoleucine	2.56
L-Methionine	4.90
L-Proline	5.02

* One unit of NADPH-cytochrome *c* reductase is the amount of enzyme catalyzing the formation of 1 µmole of reduced cytochrome *c* in 15 min.

induction of NADPH-cytochrome *c* reductase is presented in Table VII. It could be seen that NADPH-cytochrome *c* reductase is also repressed by those compounds which repress nitrate reductase. In this case also, isoleucine, glutamine and asparagine were found to be the most potent corepressors.

DISCUSSION

The nitrate reductase reaction is the first step in the assimilation of nitrate. Ammonia and the amino acids are the products of the overall reduction of nitrate to the amino level. Therefore a reaction of this importance would naturally be expected to be vigorously controlled by the organism. Many amino acids, especially glutamine and asparagine have been found to carry out this control remarkably well in *N. crassa*. Feedback inhibition by NH_4^+ and the amino acids is ruled out by the data in Table V.

FILNER[19] has studied the induction and repression of nitrate reductase in cultured tobacco cells. He observed that those amino acids which inhibit growth, also act as corepressors of nitrate reductase. In *N. crassa*, however, a different situation is observed. Growth-promotory and growth-inhibitory amino acids alike are found to repress nitrate reductase effectively (Table III). Plant nitrate reductases are generally NADH-dependent while microbial nitrate reductases are dependent on NADPH (ref. 20). Further, fungal nitrate reductases but not the plant enzymes are repressed by NH_4^+ (ref. 20). Thus it appears that the mode of regulation of nitrate reductase in microbial systems may differ from that in plants. After the reduction of nitrate to ammonia the incorporation of the ammonia into glutamic acid by the enzyme glutamate dehydrogenase is considered to be the major route for the final assimilation of the nitrogen into organic compounds in plants[21]. Surprisingly both glutamic and aspartic acids have been found to have no effect on the induction of nitrate reductase in *N. crassa*, in contrast to glutamine and asparagine. The action of glutamine and asparagine differs from that of NH_4^+. The latter fails to cause a complete abolition of the enzyme activity initially present and also permits some enzymic synthesis later on.

The complete loss of the enzyme activity present initially accompanying the repression of nitrate reductase by glutamine, asparagine, cysteine and isoleucine is an intriguing feature of the regulation of nitrate reductase in *N. crassa*. COVE observes a similar situation with nitrate reductase of *A. nidulans*[5]. He finds enzyme repression as well as loss of enzyme activity on transferring mycelia grown in sole nitrate medium to sole ammonium or ammonium *plus* nitrate media. It may be argued that the *N. crassa* nitrate reductase is labile in the absence of its substrate, nitrate, and the amino acids might act by shutting off the supply of nitrate to the cells. But the data presented in Table VI show that the amino acids and NH_4^+ do not block the uptake of nitrate by the mycelia. Therefore enzyme repression and enzyme inactivation occur in spite of a continuous supply of nitrate. Another possibility is that the enzyme may undergo continuous turnover and hence in the absence of enzyme synthesis, loss of enzyme activity would occur. INGLE, JOY AND HAGEMAN[20] have studied the inhibition by cycloheximide, puromycin and actinomycin D of nitrate reductase induction in radish cotyledons. They find that these antibiotics, in addition to inhibition of enzyme synthesis, also cause loss of enzyme activity. This they

attribute to the lability and rapid turnover of nitrate reductase in radish cotyledons. This is not found to be the case in Neurospora. Cycloheximide at a concentration of 5 μg/10 ml stops nitrate reductase induction completely in *N. crassa*. But when cycloheximide is added 1 h after the transfer of the mycelia to sole nitrate medium, the enzyme activity already induced is not lost though further enzyme synthesis is stopped (Table I). This shows that the Neurospora nitrate reductase is quite stable in the presence of an inhibitor of protein synthesis and that it does not undergo turnover. Yet another possibility is that an amino acid like glutamine may induce a protease specific for action on nitrate reductase. The addition of glutamine causes loss of enzyme activity, but surprisingly when cycloheximide is also added with glutamine after the enzyme induction has begun, the enzyme activity is lost still faster (Table I). This would rule out the induction of a protease by glutamine for the degradation of nitrate reductase.

Thus, the corepressors of nitrate reductase not only stop enzyme synthesis but also inactivate the enzyme *in vivo*. This might take place either by the spontaneous degradation of the enzyme owing to the lability introduced in it by some unknown mechanism *in vivo*, or by the conversion of the enzyme protein to an inactive form. Further studies are needed to elucidate the mode of action of these corepressors. A similar situation is observed by FERGUSON, BOLL AND HOLZER[22] in their studies on the induction of yeast malate dehydrogenase by acetate. The repression of malate dehydrogenase by glucose is found to be accompanied by enzyme inactivation. Many other instances of such a phenomenon taking place are known in fungi and this process has been termed 'inactivation-repression'[22].

The findings of KINSKY AND MCELROY[9] that nitrate reductase preparations from *N. crassa* contained an inseparable NADPH-cytochrome *c* reductase with somewhat similar properties have suggested that the two activities may be present in the same enzyme. However there is considerable 'constitutive' NADPH-cytochrome *c* reductase in non-induced cells and so it has been suggested that there are two NADPH-cytochrome *c* reductases[23], (a) an inducible enzyme associated with nitrate reductase activity, and (b) a non-inducible 'constitutive' enzyme having no nitrate reductase activity. SORGER[7] finds that β-methylaspartate and 2-methylalanine which repress nitrate reductase, also repress NADPH-cytochrome *c* reductase. Our results substantiate the earlier findings that the two activities may reside in the same enzyme. We find those corepressors which control nitrate reductase, repress NADPH-cytochrome *c* reductase also. It is suggested by NASON[23] that the two enzymes may share a common enzymatic step, presumably the rate-limiting NADPH-flavin reductase reaction.

The dependence of catalase on nitrate reductase in *N. crassa* is known[8,10]. In the present study another instance of this dependence has been provided. The induction of catalase follows the induction by nitrate of nitrate reductase in the mycelia. The amino acids which repress nitrate reductase have been found to repress catalase, with the exception of the basic amino acids, arginine, lysine and ornithine (Table III). Leucine which represses nitrate reductase only moderately and methionine and tryptophan which have only a little control on nitrate reductase are found to repress catalase effectively. This is found to be due to their action directly on catalase (Table IV). It is interesting to note the interdependence of the three enzymes, nitrate reductase, NADPH-cytochrome *c* reductase and catalase. Whereas the former

two enzyme activities are probably exhibited by a single protein[7], the catalase level, although due to a distinctly different protein, is dictated by the level of nitrate reductase. The results show that the endogenous H_2O_2 acts as an inducer for catalase and although doubts are still raised as to the exact role of catalase in living cells, one function is definitely the classical role attributed to the protein, namely the disposal of H_2O_2.

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