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THE ROLE OF MOLYBDENUM IN THE SYNTHESIS OF NEUROSPORA NITRATE REDUCTASE

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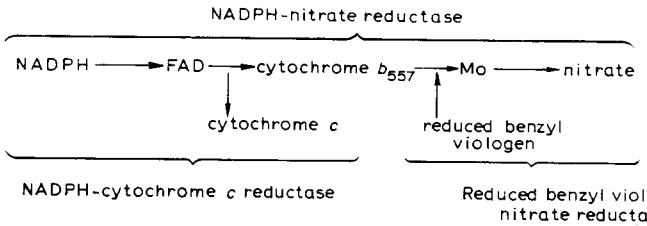
SUMMARY

The enzyme complex exhibiting NADPH–nitrate reductase activity in *Neurospora crassa* has associated with it nitrate-inducible NADPH–cytochrome *c* reductase activity and reduced benzyl viologen–nitrate reductase activity. Induction of *Neurospora mycelia* by nitrate under molybdenum-deficient or tungstate-toxic conditions results in low NADPH–nitrate reductase and reduced benzyl viologen–nitrate reductase activities, but the level of NADPH–cytochrome *c* reductase activity is the same as under normal conditions. By means of sucrose density gradient centrifugation, it is shown that the NADPH–cytochrome *c* reductase induced under molybdenum deficiency or tungstate toxicity has the same $s_{20,w}$ value as the nitrate reductase complex induced under normal conditions, but has some other properties that are different. It is proposed that during molybdenum deficiency or tungstate toxicity, the component proteins of the nitrate reductase complex are all synthesized, but owing to the non-availability of molybdenum, the molybdenum-binding site on the enzyme is folded *in vivo* in such a way that it can no longer bind this metal *in vitro*. The enzyme consequently has no nitrate reductase activity, but retains its cytochrome *c* reductase activity which does not require molybdenum.

INTRODUCTION

The enzyme NADPH–nitrate reductase (NADPH–nitrate oxidoreductase, EC 1.6.6.3) in *Neurospora crassa* is a nitrate-inducible molybdoflavoprotein which is also known to catalyze the following partial reactions involved in nitrate reduction: the reduction of cytochrome *c* by NADPH and the reduction of nitrate by reduced benzyl (or methyl) viologen[1–4]. Strong genetic and biochemical evidence has accumulated to show that Neurospora nitrate reductase is an aggregate of more than one protein, each protein component catalyzing a part of the reaction catalyzed by the whole complex. Several nitrate-nonutilizing mutants of Neurospora have been isolated. All these mutants lack NADPH–nitrate reductase activity. However, SORGER[3] and SORGER AND GILES[5] have found that the mutant *nit-1* possesses nitrate-inducible NADPH–cytochrome *c* reductase activity and that the mutant *nit-3* has reduced benzylviologon–nitrate reductase activity. The NADPH–cytochrome *c* reductase of *nit-1* (ref. 3) and the reduced benzyl viologen–nitrate reductase of *nit-3*

(ref. 6) have a lower $s_{20,w}$ value than the nitrate reductase complex of wild type mycelia. The presence of cytochrome b_{557} in the nitrate reductase complex has been demonstrated recently and the following scheme drawn by GARRET AND NASON[4] shows the components of this complex and the reactions catalyzed by them.



Molybdenum is believed to be an electron carrier in the electron-transport sequence of nitrate reduction and is essential for NADPH- and reduced benzyl viologen–nitrate reductase activities, but not for NADPH–cytochrome *c* reductase activity[1,7]. NICHOLAS AND NASON[7] found that extracts of mycelia grown under molybdenum-deficient conditions have low NADPH–nitrate reductase activity which is not restored to normal levels by the addition of molybdate *in vitro*. This observation was interpreted to indicate that the nitrate reductase apoprotein is absent from extracts of molybdate-deficient mycelia and that consequently molybdenum is necessary for the synthesis of the nitrate reductase apoprotein. However, it was demonstrated by SUBRAMANIAN AND SARMA[8] that the nitrate-inducible NADPH–cytochrome *c* reductase activity is still induced by nitrate under molybdenum-deficient conditions. This observation[8] contradicts the proposal[7] that molybdenum is necessary for the synthesis of the nitrate reductase apoproteins.

In this study two possible explanations of the effect of molybdenum deficiency are examined by making use of the sucrose density gradient centrifugation technique: (i) only that part of the nitrate reductase complex displaying cytochrome *c* reductase activity is induced; or (ii) the whole nitrate reductase complex is induced but only the component having cytochrome *c* reductase activity is functional.

MATERIALS AND METHODS

N. crassa strains 74A (wild type) and *nit-1* 6a (nitrate-nonutilizing mutant) were used in this study. The media used, growth conditions, enzyme induction and extraction prodecure were the same as described earlier[9]. *N. crassa* was grown in ammonium medium in 250 ml. Erlenmeyer flasks in stationary cultures at 27° for 40 h. The mycelia were washed with water, transferred to nitrate medium and shaken in a rotary shaker at 27° for 2.5 h. They were then harvested, extracted with cold 0.1 M potassium phosphate buffer (pH 7.0) and centrifuged in the cold at 5000 × *g* for 10 min. The supernatant was used as the enzyme preparation.

Production of molybdenum deficiency and tungstate toxicity

Media were made molybdate-deficient by means of the CuS co-precipitation method[10]. Tungstate toxicity was produced by including sodium tungstate in the medium at a final concentration of 6 mM.

Enzyme assays

NADPH–nitrate reductase and NADPH–cytochrome *c* reductase activities were assayed as described earlier[9].

Reduced benzyl viologen–nitrate reductase activity was assayed as follows: the reaction mixture consisted of 0.25 ml of 0.1 M potassium phosphate buffer (pH 7.0); 0.10 ml of 1 mM benzyl viologen; 0.10 ml of 0.1 M $NaNO_3$; 0.05 ml of a freshly prepared solution of $Na_2S_2O_4$ (8 mg/ml of 0.8 % $NaHCO_3$), and enzyme. The reaction was started by the addition of dithionite and after 10 min it was stopped by agitating the tubes vigorously in a Vortex mixer until the violet colour of the dye had completely disappeared. Then 3 ml of water, 0.5 ml of 1 % sulphanilamide in 1 M HCl and 0.5 ml of aqueous 0.02 % α-(*N*-1)-naphthyl ethylenediamine dihydrochloride were added, and after 5 min, the colour of the azo dye produced was measured at 540 nm in a Beckman DB spectrophotometer. Protein was estimated by the biuret method[11].

Units of enzyme activity

1 unit of NADPH–nitrate reductase and reduced benzyl viologen–nitrate reductase is defined as the amount of enzyme that catalyzes the formation of 1 nmole of nitrite per min.

1 unit of NADPH–cytochrome *c* reductase is defined as the amount of enzyme that catalyzes the formation of 1 nmole of reduced cytochrome *c* per min.

Specific activity is expressed as units/mg protein.

Sucrose density gradient centrifugation

The procedure was essentially the same as that described by MARTIN AND AMES[12]. Samples of 0.2 ml of the extracts to be examined were layered on top of linear, precooled 5–20 % sucrose density gradients in 0.1 M phosphate buffer (pH 7.0). The tubes were centrifuged at 117000 × *g* for 14 h at 5° in a swinging bucket rotor (Spinco SW 50L) in a refrigerated Spinco Model L centrifuge. Fractions of 7 drops each were collected by downward gravity flow through a hypodermic needle. Commercially prepared catalase was used as the marker to determine the $s_{20,w}$ values of NADPH–nitrate reductase and NADPH–cytochrome *c* reductase in the gradients.

RESULTS

The results presented in Table I show the levels of NADPH–nitrate reductase, NADPH–cytochrome *c* reductase and reduced benzyl viologen–nitrate reductase activities under various conditions. The NADPH–cytochrome *c* reductase activity present in uninduced mycelia is due to a constitutive enzyme which is present under all conditions of growth and is unrelated to NADPH–nitrate reductase[3,13]. Induction by nitrate under normal (molybdenum-sufficient) conditions results in high levels of NADPH–nitrate reductase, NADPH–cytochrome *c* reductase and reduced benzyl viologen–nitrate reductase activities. Induction under molybdenum deficient or tungstate-toxic conditions results in much lowered levels of NADPH–nitrate reductase and reduced benzyl viologen–nitrate reductase activities, while the NADPH–cytochrome *c* reductase activity is induced to the same extent as under normal conditions. Tungstate is known to be an antagonist of molybdate and produces conditions

TABLE I

ENZYME ACTIVITIES UNDER VARIOUS CONDITIONS OF INDUCTION

Wild-type or *nit-1* Neurospora mycelia were grown in ammonium medium at 27° for 2 days. The mycelia were washed and transferred to nitrate media which were normal, molybdenum-deficient or tungstate-toxic. After 2.5 h of induction with shaking, the mycelia were washed and extracted with 0.1 M phosphate buffer (pH 7.0) using the same volume of extraction buffer in each case. The enzymes were assayed as described in MATERIALS AND METHODS.

State of induction	*Enzyme specific activity (units/mg protein)*				*Total yield of protein (mg)*	
	Wild type			*nit-1*		
	NADPH–nitrate reductase	*NADPH–cytochrome c reductase*	*Reduced benzyl viologen–nitrate reductase*	*NADPH–cytochrome c reductase*	*Wild type*	*nit-1*
Uninduced	0	28.0	0	22.0	16.6	13.0
Induced, normal	13.20	116.8	0.23	81.0	20.2	14.6
Induced, molybdenum-deficient	4.23	106.0	1.25	84.5	19.0	14.2
Induced, tungstate-toxic	1.86	108.5	0.83	92.3	18.4	15.0

similar to molybdenum deficiency in *N. crassa*[14] and barley[15]. The *nit-1* mutant possesses nitrate-inducible NADPH–cytochrome *c* reductase activity but lacks NADPH–nitrate reductase and reduced benzyl viologen–nitrate reductase activities[3]. The induction of NADPH–cytochrome *c* reductase activity in *nit-1* is found to be unaffected by molybdenum deficiency or tungstate toxicity (Table I).

Since NADPH–cytochrome *c* reductase activity was induced by nitrate in wild-type mycelia under molybdate-deficient or tungstate-toxic conditions, the question arose whether this cytochrome *c* reductase represents only one component of the nitrate reductase complex in which case it should have the same size as the cytochrome *c* reductase induced by nitrate in *nit-1* (refs. 3 and 6); or whether it represents the entire protein complex of nitrate reductase in which case it should

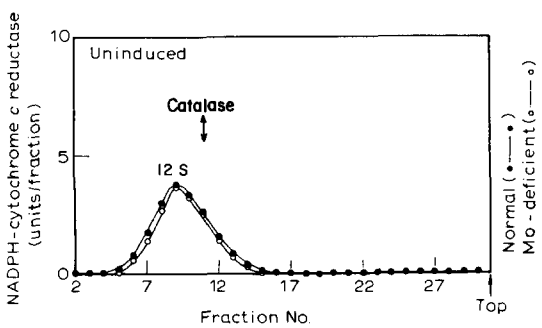


Fig. 1. NADPH–cytochrome *c* reductase activity profiles of 5–20% sucrose density gradients through which extracts of uninduced wild-type mycelia grown under normal (●—●) and molybdenum-deficient (○—○) conditions have been centrifuged. A total of 31 fractions of 7 drops each were collected from the gradients. Units of activity layered on the gradients: cytochrome *c* reductase; normal, −54; molybdenum-deficient, 51. Recoveries of activity are 35.2 and 33.9%, respectively.

have the same size as the NADPH–cytochrome *c* reductase or NADPH–nitrate reductase induced in wild-type mycelia under normal conditions. To differentiate between these two possibilities, the extracts of mycelia induced under various

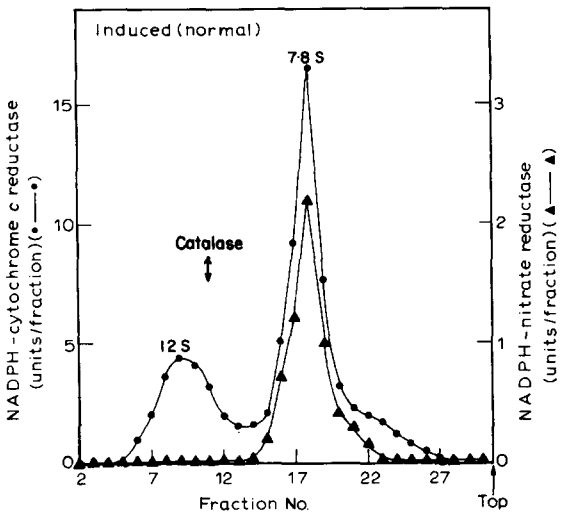


Fig. 2. NADPH–cytochrome *c* reductase (●—●) and NADPH–nitrate reductase (▲—▲) activity profiles of 5–20% sucrose densit gradients through which fresh extracts of wild-type mycelia induced by nitrate under normal conditions have been centrifuged. 0.2 ml of the extract containing 230 units of cytochrome *c* reductase activity and 26.5 units of nitrate reductase activity was layered on the gradients. A total of 32 fractions of 7 drops each were collected from the gradients. Recoveries of activity are 32.5 and 22% for cytochrome *c* reductase and nitrate reductase, respectively.

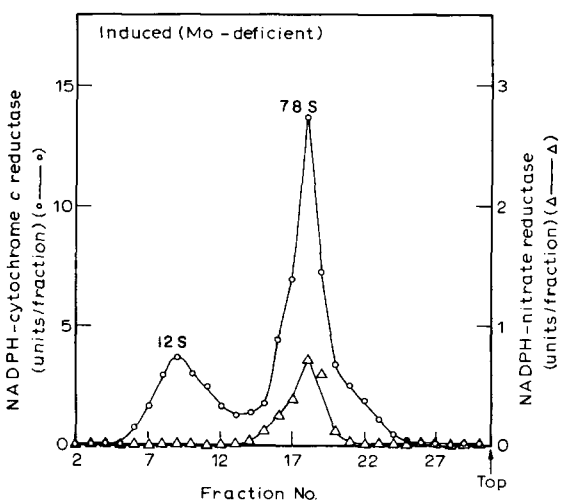


Fig. 3. NADPH–cytochrome *c* reductase (○—○) and NADPH–nitrate reductase (△—△) activity profiles of 5–20% sucrose density gradients through which fresh extracts of wild-type mycelia induced by nitrate under molybdenum-deficient conditions have been centrifuged. 0.2 ml of the extract containing 210 units of cytochrome *c* reductase activity and 8.5 units of nitrate reductase activity was layered on the gradients. A total of 32 fractions of 7 drops each were collected from the gradients. Recoveries of activity are 29.7 and 26.7% for cytochrome *c* reductase and nitrate reductase, respectively.

conditions were subjected to sucrose density gradient centrifugation (Figs. 1–5). The peak of NADPH–cytochrome *c* reductase acrivity with an $s_{20,w}$ value of 12.0 seen in all these figures is the ubiquitous constitutive cytochrome *c* reductase which is

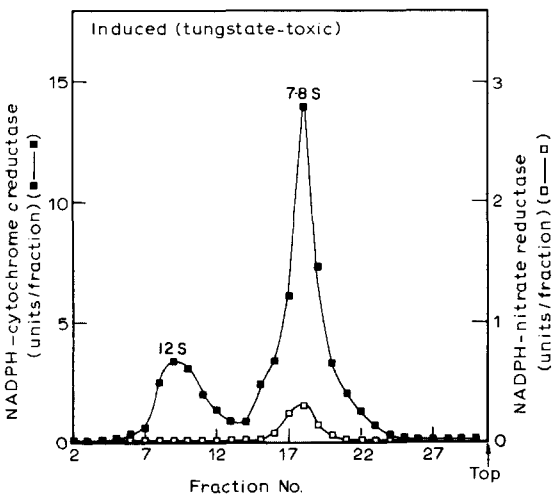


Fig. 4. NADPH–cytochrome *c* reductase (■—■) and NADPH–nitrate reductase (□—□) activity profiles of 5–20% sucrose density gradients through which fresh extracts of wild-type mycelia induced under tungstate-toxic conditions have been centrifuged. 0.2 ml of the extract containing 217 units of cytochrome *c* reductase activity and 3.9 units of nitrate reductase activity was layered on the gradients. Recoveries of activity are 25.8 and 22.6% for cytochrome *c* reductase and nitrate reductase, respectively.

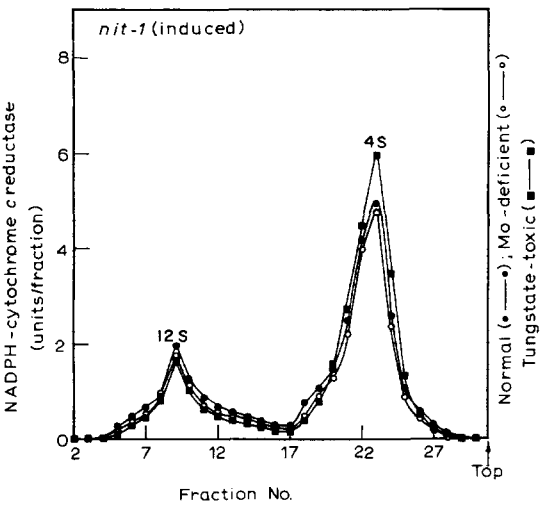


Fig. 5. NADPH–cytochrome *c* reductase profiles of 5–20% sucrose density gradients through which fresh extracts of *nit-1* mycelia induced by nitrate under normal (●—●), molybdenum-deficient (○—○), and tungstate-toxic (■—■) conditions, have been centrifuged. A total of 32 fractions of 7 drops each were collected from the gradients. Units of activity layered on the gradients: normal, 121; molybdenum-deficient, 126; tungstate-toxic, 138. Recoveries of activity are 23.9, 20.6 and 21.0%, respectively.

unrelated to the nitrate reductase complex[3]. As expected, this constitutive cytochrome *c* reductase activity is unaffected by molybdenum deficiency (Fig. 1).

The nitrate reductase complex from wild-type mycelia induced under normal conditions, which exhibits both NADPH–nitrate reductase and NADPH–cytochrome *c* reductase activities, has an $s_{20,w}$ value of 7.8 (Fig. 2). The NADPH–cytochrome *c* reductase induced in wild-type mycelia under molybdenum deficiency (Fig. 3) or tungstate toxicity (Fig. 4) also has an $s_{20,w}$ value of 7.8. Thus it can be seen that the NADPH–cytochrome *c* reductase induced in wild-type mycelia under molybdenum deficiency or tungstate toxicity is of the same size as the nitrate reductase complex induced under normal conditions, and is larger than the NADPH–cytochrome *c* reductase induced by nitrate in *nit-1* which has an $s_{20,w}$ value of 4.0 (Fig. 5). The extent of formation of both the constitutive ($s_{20,w} = 12$) and the induced ($s_{20,w} = 7.8$) NADPH–cytochrome *c* reductases is unaffected by molybdenum deficiency or tungstate toxicity (Figs. 2–4). On the other hand, the nitrate reductase activity induced in wild-type mycelia under molybdenum deficiency (Fig. 3) or tungstate toxicity (Fig. 4) is much lower than that induced under normal conditions (Fig. 2). The NADPH–cytochrome *c* reductase induced in *nit-1* is unaffected by molybdenum deficiency or tungstate toxicity (Fig. 5 and Table I).

The above results show that the NADPH–cytochrome *c* reductase induced by nitrate in wild-type mycelia under molybdenum deficiency or tungstate toxicity has the following characteristics: (i) greatly reduced NADPH–nitrate reductase activity, (ii) the same $s_{20,w}$ value as that of the nitrate reductase complex induced under normal conditions, and (iii) a higher $s_{20,w}$ value than that of the NADPH–cytochrome *c* reductase induced by nitrate in *nit-1*.

The heat inactivation kinetics of NADPH–cytochrome *c* reductase activity in crude extracts of nitrate-induced *N. crassa* mycelia is known to give rise to a biphasic curve, due to the presence of two species of cytochrome *c* reductases, one being constitutive and the other inducible. The constitutive cytochrome *c* reductase

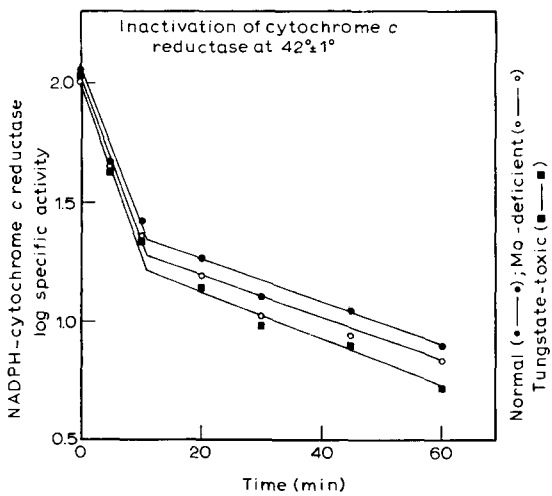


Fig. 6. Effect of exposure to 42 ± 1° on NADPH–cytochrome *c* reductase activity present in extracts of wild-type mycelia induced by nitrate under normal (●—●), molybdenum-deficient (○—○), and tungstate toxic (■—■) conditions.

TABLE II

EFFECT OF OVERNIGHT STORAGE IN THE COLD ON NADPH–CYTOCHROME *c* REDUCTASE ACTIVITY

Enzyme extracts were prepared as described in Table I. Enzyme assays were made with fresh extracts as well as with extracts kept at 5° overnight.

Extracts	*Specific activity of NADPH–cytochrome c reductase (units/mg protein)*		*Specific activity of NADPH–nitrate reductase (units/mg protein)*		*Total yield of protein (mg)*
	Fresh extract	*Extract subjected to overnight cold storage*	*Fresh extract*	*Extract subjected to overnight cold storage*	
Uninduced	30.3	28.6	0	0	18.4
Induced, normal	120.0	114.5	13.6	12.1	23.0
Induced, molybdenum-deficient	104.3	126.7	4.1	3.5	22.2
Induced, tungstate-toxic	106.8	135.3	1.8	1.5	21.6

is known to be much less heat-sensitive than the inducible one[16]. In the present study, it is found that the NADPH–cytochrome *c* reductases induced in wild-type mycelia under normal, molybdate-deficient or tungstate-toxic conditions all follow the same heat inactivation kinetics at 42 ± 1° (Fig. 6). Each curve is biphasic showing the presence of two species of cytochrome *c* reductase in each extract.

Extracts of wild-type mycelia induced under molybdenum-deficient or tungstate-toxic conditions, but not of those induced under normal conditions, show an increase in cytochrome *c* reductase activity after being kept at 5°C overnight (Table II). The NADPH–nitrate reductase activity of these extracts decreases slightly as a result of this treatment. The cytochrome *c* reductase induced in tungstate-toxic conditions is found to have a slightly higher pH optimum than that induced under normal conditions in wild-type mycelia (Fig. 7). These observations suggest that there are

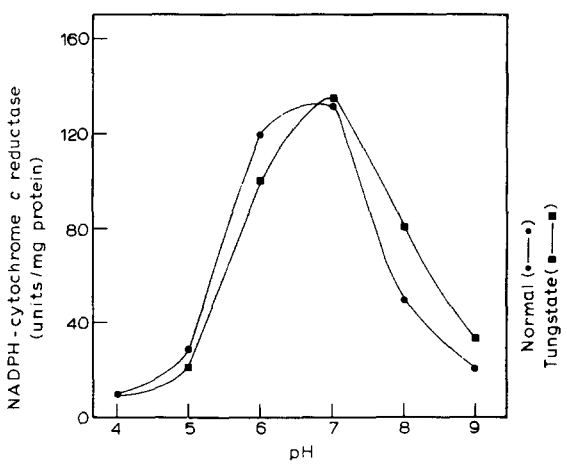


Fig. 7. Effect of pH on NADPH–cytochrome *c* reductase activity of fresh extracts of wild-type mycelia induced by nitrate under normal (●—●) or tungstate-toxic (■—■) conditions. 0.1 M citrate–phosphate buffer and 0.1 M Tris–HCl buffer were used for the pH ranges 4–7 and 8–9, respectively.

differences between the cytochrome *c* reductase induced under normal conditions on the one hand and the cytochrome *c* reductase induced under molybdenum deficiency or tungstate toxicity on the other hand.

The NADPH–cytochrome *c* reductases in extracts of wild-type mycelia induced under normal, molybdenum-deficient or tungstate-toxic conditions have the same K_m values for cytochrome *c* and NADPH. These are 35 μM and 15 μM for cytochrome *c* and NADPH, respectively.

The reduction of cytochrome *c* by Neurospora NADPH–nitrate reductase is known to be inhibited by nitrate, the other substrate of this enzyme. This is most probably due to a competition for reducing equivalents between nitrate and cytochrome *c*. The NADPH–cytochrome *c* reductase activity induced under molybdenum-deficient or tungstate-toxic conditions is found to be much less sensitive to inhibition by nitrate than that induced under normal conditions (Table III).

TABLE III

INHIBITION OF NADPH–CYTOCHROME *c* REDUCTASE ACTIVITY BY NITRATE *in vitro*

Nitrate was added at a final concentration of 5 mM to the reaction mixture for the assay of cytochrome *c* reductase activity.

Enzyme extracts	*NADPH–cytochrome c reductase (units/mg protein)*	
	No nitrate	*5 mM nitrate*
Induced, normal	110	65
Induced, molybdate-deficient	102	90
Induced, tungstate-toxic	105	100

DISCUSSION

The nitrate-induced NADPH–cytochrome *c* reductase of the Neurospora mutant *nit-1* is known to band in sucrose density gradients with an $s_{20,w}$ value much lower than that of the nitrate reductase complex of wild-type and equal to that of the component of wild-type nitrate reductase complex displaying cytochrome *c* reductase activity which is obtained by steapsin treatment of this complex[3]. The results from density gradient centrifugation experiments in the present study show that the NADPH–cytochrome *c* reductase induced by nitrate in wild-type mycelia under molybdenum-deficient or tungstate-toxic conditions possesses only one of the catalytic activities (*viz.* the cytochrome *c* reductase activity) of the nitrate reductase complex induced under normal conditions, but has the same size as the whole nitrate reductase complex and is larger than the NADPH–cytochrome *c* reductase induced by nitrate in the mutant *nit-1*. The simplest interpretation of these results is that molybdenum is not necessary for the synthesis of the protein components of nitrate reductase and that under molybdenum-deficient or tungstate-toxic conditions the whole nitrate reductase aggregate is synthesized, with the component exhibiting cytochrome *c* reductase activity being enzymatically active and the other component(s) being enzymatically inactive.

WRAY AND FILNER[15] detected three peaks of NADH–cytochrome *c* reductase activity in extracts of nitrate-induced barley shoots by sucrose density gradient centrifugation analysis. One of these, sedimenting near the bottom of the gradients was due to a constitutive NADH-cytochrome *c* reductase and is equivalent to the 12-S NADPH–cytochrome *c* reductase of Neurospora (Figs. 1–5). The other two, having $s_{20,w}$ values of 8.0 and 3.7 were nitrate-inducible. The NADH-cytochrome *c* reductase having an $s_{20,w}$ value of 8.0 had associated NADH–nitrate reductase activity and is equivalent to the nitrate reductase complex of Neurospora. The cytochrome *c* reductase activity having an $s_{20,w}$ value of 3.7 has no obvious equivalent in our system. Tungstate toxicity caused a marked lowering of nitrate reductase activity but gave rise to a super-induction of cytochrome *c* reductase activities sedimenting with $s_{20,w}$ values of 8.0 and 3.7. However, the exact mechanism by which tungstate exerts its toxic effects on nitrate reductase are not known. It could act by, (a) getting incorporated into nitrate reductase in place of molybdenum and making the enzyme inactive; (b) blocking the uptake of molybdenum by the mycelia; or (c) inhibiting the incorporation of molybdenum into nitrate reductase, thereby creating a condition of molybdenum deficiency. Therefore we chose to investigate the role of molybdenum in the synthesis of Neurospora nitrate reductase directly by observing the effect of molybdenum deficiency – by the removal of the metal from the culture medium – on the size and some other properties of nitrate reductase.

It is known that the addition of molybdate *in vitro* to extracts of wild-type mycelia induced by nitrate under molybdenum-deficient conditions does not result in the restoration of nitrate reductase activity[7]. This can be explained by proposing that when nitrate reductase is synthesized under molybdate-deficient or tungstate-toxic conditions, the enzyme folds in such a way that it can no longer bind molybdenum when exposed to this metal. Such an altered enzyme would probably lack nitrate reductase activity but would retain its cytochrome *c* reductase activity, as the latter activity does not require molybdenum.

The other findings made in this study are: (a) the NADPH–cytochrome *c* reductase activity of extracts of wild type mycelia induced under molybdenum-deficient or tungstate-toxic conditions, but not of those induced under normal conditions, increases after overnight exposure to cold; (b) the cytochrome *c* reductase activity induced under molybdenum-deficient or tungstate-toxic conditions is much less sensitive to inhibition by nitrate *in vitro* than that induced under normal conditions; and (c) the cytochrome *c* reductase activity induced under tungstate-toxic conditions has a higher pH optimum than that induced under normal conditions. The observations suggest possible structural differences between the nitrate reductase complex induced under normal conditions and the cytochrome *c* reductase induced under molybdate-deficient or tungstate-toxic conditions in wild-type mycelia.

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