Multiplicity of Hydroxylamine Reductase Activities in Neurospora crassa

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Enzymes capable of catalyzing the hydroxylamine-dependent oxidation of pyridine nucleotides have been studied in Salmonella typhimurium (1, 2), Escherichia coli (3–5), Bacillus subtilis (6, 7), Azotobacter agile (8, 9), Neurospora crassa (10), and soybean leaves (11). Siegel, Click, and Monty (1) and Siegel and Monty (2) demonstrated that reduced triphosphopyridine nucleotide-specific hydroxylamine reductase activity found in soluble extracts of S. typhimurium is entirely identified with the enzyme sulfite reductase (HS−NADP oxidoreductase, EC 1.8.1.2). Thus, the level of hydroxylamine reductase varies in a coordinate fashion with the level of sulfite reductase when different sulfur sources are utilized for growth of the bacteria, both activities being completely repressed on cysteine-containing media. The integrity of six cistrons is required for maintenance of the ability to reduce sulfite to sulfide in Salmonella (12); a loss of function in any one of these cistrons leads to loss of all TPNH-hydroxylamine reductase activity in extracts (1). Competition experiments have implicated a common enzymatic site for the reduction of both sulfite and hydroxylamine (2). A constant ratio of activities with the two substrates was obtained throughout a partial purification of Salmonella sulfite reductase.¹ Mager (3), Lazzarini and Atkinson (4), and Kemp, Atkinson, Ehret, and Lazzarini (5) have presented evidence which strongly indicates the identity of the hydroxylamine and sulfite reductase activities of E. coli.

In contrast to the results in bacteria, in which organisms hydroxylamine reduction appears to be an incidental property of an enzyme concerned with sulfur metabolism, Zucker and Nason (10) have described a pyridine nucleotide-dependent hydroxylamine reductase from N. crassa whose level is "adaptive" to the presence of nitrate in the growth medium. The enzyme was active with either DPNH or TPNH and was inhibited by sulfite. The possible connection of this enzyme with the pathway of nitrate assimilation in Neurospora was cast in doubt, however, when Zucker and Nason (10) demonstrated that the enzyme catalyzing the production of 1 μmole of TPNH per min at 25° in the reaction mixture described below for the assay of sulfite reductase.

Growth of Mycelia and Preparation of Extracts—The sources and nutritional properties of the various strains of N. crassa employed in these experiments have been described previously (14). Mycelia were grown in 3-liter batches with forced aeration at room temperature (22–25°) for 40 to 52 hours, with the exception of mycelia prepared for density gradient centrifugation experiments, which were grown for the time intervals indicated in the text. The media were replaced by preparing the sulfoxates of the Difeo choline assay medium (16) with the corresponding chloride salts in equimolar amounts with respect to the cation. Sulfur was supplied as sodium thiosulfate (for wild-type and for strain S0702Ra in certain experiments), sodium sulfate (for strains P22a and ααD-1), or α-thionine (for other strains) at a final concentration of 2.4 × 10⁻⁶ M. The thiosulfate and methionine solutions were filter-sterilized through Pyrex sintered glass filters (ultrasonde) and added aseptically to the sterile medium.

Mycelia were harvested by filtration, washed for 10 to 20 min at room temperature in 0.06 M sodium phosphate buffer, pH 7.0, and quickly washed with distilled water. Mycelia thus obtained were frozen in thin sheets at −15°. Extracts were prepared by grinding the frozen mycelia with small amounts of sand and 0.04 M sodium phosphate buffer, pH 8.0, containing 0.005 M EDTA. The supernatant fluid, obtained by centrifuga-

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¹ L. M. Siegel and K. J. Monty, unpublished data.
tion at 30,000 × g for 30 min was subjected to a second centrifugation at 109,000 × g for 90 min in the Spinco, model L, ultracentrifuge with a No. 50 rotor. The supernatant thus obtained was filtered through glass wool to remove lipid, and the filtrate was subjected to a second centrifugation at 30,000 × g for 30 min. The eluate served as the enzyme preparation for subsequent experimentation. For certain density gradient centrifugation experiments, indicated in the text, the 109,000 × g supernatant was utilized without dialysis on Sephadex G-25 columns.

Assay of Hydroxylamine Reductase—Enzyme activity was determined spectrophotometrically by following the change in absorbance at 340 μm on oxidation of TPNH. All measurements were made with a Beckman model DU spectrophotometer with cuvettes having a light path of 1 cm. The reaction mixture contained the following in 1-ml total volume: sodium pyrophosphate, pH 8.0, 65 mM; FAD, 3.3 × 10⁻⁴ mM; TPNH, 0.2 mM; hydroxylamine hydrochloride, 13.3 mM; and an appropriate amount of enzyme. The endogenous rate of TPNH oxidation was observed for 1 to 2 min before addition of hydroxylamine to the otherwise complete reaction mixture. Subsequent to addition of substrate, the rate was followed for an additional 2 to 5 min. All rates have been corrected for any endogenous activity, the latter usually being negligible. The oxidation of TPNH was proportional to time during the intervals studied in these experiments.

Assay of Sulfite Reductase—Enzyme activity was measured by the production of sulfide from sulfite, the former being determined as methylene blue by the method of Siegel (17). Reaction mixtures contained the following in 1-ml total volume: sodium phosphate, pH 8.0, 80 mM; FAD, 0.45 mM; TPN, 0.2 mM; glucose 6-phosphate, 5 mM; sodium bisulfite, 0.5 mM; enzyme; and glucose 6-phosphate dehydrogenase, 0.06 unit. Absorbance at 340 μm upon disappearance of peroxide, according to the method of Beers and Sizer (18), was determined spectrophotometrically by following the change in absorbance at 340 μm upon disappearance of peroxide, according to the method of Beers and Sizer (18). Protein was determined by the biuret method described by Zamenhof (19), with bovine plasma albumin as standard.

Density Gradient Centrifugation—Sucrose gradients of 4.5 ml each were prepared in cellulose nitrate tubes from 15.5 and 33% (w/v) sucrose solutions in 0.04 M sodium phosphate, pH 8.0, containing 0.005 M EDTA. The device employed for the preparation of the gradients was designed by Dr. T. P. Salo. The gradients were kept in the cold for from 8 to 36 hours prior to use. Linearity of the sucrose gradients was determined by addition of phenol red to the sucrose solution of lower concentration; after centrifugation and collection of fractions, the absorbance at 400 μm was found to increase linearly with fraction number.

In experiments involving centrifugation of extracts, 0.01 ml of catalase was added to 0.25 ml of a 109,000 × g supernatant (dialyzed or undialyzed, as indicated below), and 0.2 ml of this mixture was layered onto the gradient. Centrifugation was performed at 39,000 rpm for 17 to 18 hours in the Spinco, model L, ultracentrifuge with a No. 39SW rotor. Fractions ranging from 54 to 76 in number were collected and assayed for catalase, hydroxylamine reductase, and sulfite reductase. The results of variations in total number of fractions collected from one experiment to another, results are presented in terms of movement of activity relative to catalase (designated R), rather than fraction number, for ready comparison of different experiments. Movement relative to catalase is defined by the equation, \( R = (T - X)/(T - C) \), where \( T \) = total number of fractions, \( X \) = fraction number of peak activity, and \( C \) = fraction number of maximum catalase activity.

Results and Discussion

Variation of Activities with Stage of Growth—Leinweber and Monty (14) have reported recently that the level of sulfite reductase in Neurospora is highly dependent on the stage of growth of the mycelium. Because of this observation, the possibility of a constant ratio of the levels of hydroxylamine and sulfite reductases in mycelia harvested at successive time intervals after inoculation was examined. Minimal medium supplemented with thiosulfate, 17 liters, was inoculated with 6.4 × 10⁴ conidia of wild-type strain 5297a. Growth was allowed to proceed with forced aeration at 22°. Mycelial aliquots were harvested at intervals during the period from 32 to 91 hours after inoculation. Dialyzed extracts were prepared from these mycelia as described above, and the preparations were quickly assayed for hydroxylamine and sulfite reductase activities. The variation of TPNH-hydroxylamine reductase with time of harvest is indicated in Fig. 1A, while that of sulfite reductase is shown in Fig. 2. It is evident that the level of hydroxylamine reductase falls more slowly than that of sulfite reductase as the culture matures. The correlation between the two activities in the “fresh” extracts is evidently quite poor, as shown in Fig. 3A, with the ratio of hydroxylamine to sulfite reductase varying over a 5-fold range as growth proceeds.

Zucker and Nason (10) reported that their hydroxylamine reductase preparations were unstable after dialysis against phosphate buffer at pH 8.0; furthermore, they reported a slow loss of activity on storage of the undialyzed enzyme at 15°. The dialyzed extracts from the growth experiment described above were stored frozen at −15° for 30 days, after which time they were assayed again for sulfite and hydroxylamine reductase. It was found that sulfite reductase had remained quite stable during the period of storage (Fig. 2), the minimum recovery of activity in the various extracts being 92%. The activity of TPNH-hydroxylamine reductase had dropped markedly, however, with recoveries varying from 16 to 49% (Fig. 1B). When the levels of hydroxylamine and sulfite reductases were compared in the month-old extracts, a linear relationship between the activities was now found (Fig. 3B), in sharp contrast to the previous results with fresh material.

Survey of Mutants—Leinweber and Monty (14) have described properties of a series of Neurospora mutants which lack the ability to grow on sulfate. They found that the presence of the...
enzyme sulfite reductase was always correlated with the ability of a given mutant to grow on sulfite. When freshly prepared extracts of a number of these mutants were assayed for hydroxylamine and sulfite reductase, the correlation between the two activities was found to be poor. Mutant 80702Ra (which lacks the ability to grow on sulfite), for example, possessed a higher level of hydroxylamine reductase activity than did the wild type grown and harvested under similar conditions, even though sulfite reductase was absent from the mutant. When the dialyzed extracts of the mutants were stored frozen and re-assayed from 1 to 4 weeks after preparation, an excellent correlation between the level of hydroxylamine reductase and ability to grow on sulfite was found (Table I).

That dialysis is essential for the loss of hydroxylamine reductase activity in mutants lacking sulfite reductase is demonstrated in Table II. A freshly prepared 109,000 × g supernatant of mutant 80702Ra exhibited identical levels of hydroxylamine reductase with or without dialysis. Sulfite reductase was completely absent. The extracts were stored frozen for 1 month, then assayed again for hydroxylamine reductase. Only 10% of the activity had disappeared during storage in the undialyzed preparation, whereas 90% of the activity was gone in the dialyzed aliquot. The activity in the dialyzed, stored extract could not be restored by addition of a boiled supernatant of the undialyzed extract.

**Multiplicity of Hydroxylamine Reductases**—The data presented above indicated the existence of at least two forms of hydroxylamine reductase in *Neurospora* extracts. One form, stable to dialysis, seems to be identical with the enzyme sulfite reductase in that it is present only in those strains which are capable of growing on sulfite and its level correlates well with that of sulfite reductase when the level of the latter activity is allowed to vary over a 12-fold range as a function of time of harvest of the mycelium. A second form, unstable after dialysis, constitutes virtually all of the hydroxylamine reductase activity found in mutants which are unable to grow on sulfite (99% in mutant 80702Ra). The variation in level of this second enzyme as a function of stage of growth of the mycelium may be determined by subtracting the hydroxylamine reductase activity in an aged dialyzed extract (Fig. 1B) from that in the same extract immediately following preparation (Fig. 1A). The result of this operation is shown in Fig. 1C. The "unstable" activity is seen to vary with harvest time of mycelium in a fashion independent of the variation of sulfite reductase.

To provide direct evidence for a multiplicity of hydroxylamine reductase activities in *Neurospora*, density gradient centrifugation was performed...
**Table 1**

Hydroxylamine and sulfite reductase in dialyzed, aged extracts of *Neurospora* mutant strains

Mycelia were grown in 3-liter batches with forced aeration at 22-25°C for 40 to 52 hours on minimal medium containing as sole sulfur source either sodium thiosulfate (for wild-type strain 5297a), sodium sulfite (for strains P22a and ox-D-1), or dl-methionine (for all other strains). Extracts were prepared as described in the text, dialyzed by passage through a column of Sephadex G-25, and then stored at -15°C for 1 to 4 weeks. After thawing, the preparations were assayed for sulfite and hydroxylamine reductase. Assay conditions described in the text.

<table>
<thead>
<tr>
<th>Genetic locus and strain</th>
<th>Growth on sulfite</th>
<th>Sulfite reductase (A)*</th>
<th>Hydroxylamine reductase (B)**</th>
<th>Ratio of B to A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5297a</td>
<td></td>
<td>2.6</td>
<td>39</td>
<td>15</td>
</tr>
<tr>
<td>Cys-1</td>
<td></td>
<td>0.6</td>
<td>14</td>
<td>23</td>
</tr>
<tr>
<td>Cys-2</td>
<td></td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>38401A</td>
<td></td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>460109a</td>
<td></td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>K7a</td>
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<td>0</td>
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<td></td>
</tr>
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<td>Cys-3</td>
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<td>0.8</td>
<td>17</td>
<td>21</td>
</tr>
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<td>P22a</td>
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<td>1</td>
<td></td>
</tr>
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<td>Cys-4</td>
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<td>3</td>
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<td>P1a</td>
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<td>K7a</td>
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<td></td>
</tr>
<tr>
<td>Cys-5</td>
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<td>15</td>
</tr>
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</tr>
<tr>
<td>85518A</td>
<td></td>
<td>2.7</td>
<td>46</td>
<td>16</td>
</tr>
</tbody>
</table>

* Sulfite reductase activity is expressed as millimicromoles of H₂S per min per mg of protein.
** Hydroxylamine reductase activity is expressed as millimicromoles of TPNH oxidized per min per mg of protein.

It is evident that Peak B represents the unstable hydroxylamine reductase. Only two peaks of hydroxylamine reductase activity were now discernible (Fig. 5), these peaks appearing at R = 1.15 and 0.37 in the 39-hour extract and at R = 1.07 and 0.31 in the 72-hour material, corresponding to Peaks A and C, respectively. Peak B had thus disappeared as a separate entity. When the activity at the height of each peak in the dialyzed, aged extract was compared with the analogous maximal peak activity in the undialyzed extract, recoveries were as follows for the 39-hour and 72-hour extracts, respectively: A, 92 and 98%; B, 10 and 2%; C, 85 and 73%. The hydroxylamine reductase activity at R = 0.37 was used to measure Peak R activity in all samples, and the activities have been corrected for the differing amounts of protein used for centrifugation in the dialyzed and undialyzed extracts.

It is evident that Peak B represents the unstable hydroxylamine reductase found in *Neurospora* extracts. Thus, the poor correlation between hydroxylamine and sulfite reductases found in Fig. 1A may be explained by a variation in the relative amounts of Peak A and B activities as growth proceeds. A third activity, designated Peak C, is relatively stable following dialysis and is quite small in magnitude in relation to the other hydroxylamine reductase activities. The presence of this third activity explains the displacement from the origin of the hydroxylamine and sulfite reductase activities.

**Table II**

Stability of hydroxylamine reductase in extracts of *Neurospora* mutant 80702Ra

Mycelia from mutant 80702Ra were grown with forced aeration at 23°C for 42 hours on minimal medium containing thiosulfate as the sole sulfur source. The extract was prepared as described in the text. One aliquot, which had not been dialyzed, was assayed for hydroxylamine reductase immediately after preparation. A second aliquot was dialyzed by passage through a column of Sephadex G-25, following which it was assayed immediately for hydroxylamine reductase. Both aliquots were stored at -15°C for 1 month, and, upon thawing, were reasayed for hydroxylamine reductase. Assay conditions as described in the text.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hydroxylamine reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undialyzed</td>
<td>167</td>
</tr>
<tr>
<td>Fresh</td>
<td>100</td>
</tr>
<tr>
<td>Stored</td>
<td>2</td>
</tr>
<tr>
<td>Dialyzed</td>
<td>162</td>
</tr>
<tr>
<td>Fresh</td>
<td>2</td>
</tr>
<tr>
<td>Stored</td>
<td>2</td>
</tr>
</tbody>
</table>
droxylamine against sulfite reductase plot in dialyzed, aged extracts (Fig. 3A). Thus, a very small residual hydroxylamine reductase activity is found even in aged extracts free of sulfite reductase; this activity may be attributed to Peak C.

Peak Distribution in Mutant 80702Ra—The possibility remained that all or part of the unstable hydroxylamine reductase activity of mutants which would not grow on sulfite represented an altered form of sulfite reductase rather than the Peak B activity. In order to eliminate this possibility, a nondialyzed extract of mutant 80702Ra was subjected to density gradient centrifugation. Fractions were analyzed for catalase and TPNH, and DPNH-hydroxylamine reductase. It may be seen from the data of Fig. 6 that only two peaks of hydroxylamine reductase, at \( R = 0.82 \) and 0.34, were found. The hydroxylamine reductases in both peaks were capable of oxidizing DPNH as well as TPNH, indicating the identity of these activities with Peaks B and C in wild-type Neurospora.

General Conclusions—The presence of three species of hydroxylamine reductase whose relative activities vary as the growth of the Neurospora culture proceeds seems to resolve the apparent discrepancy between the properties of hydroxylamine reductase in bacteria, in which organisms the activity appears to be an incidental property of an enzyme concerned with sulfur metabolism (1-5), and in Neurospora, where the activity described by Zucker and Nason (10) appears as an adaptation to growth on nitrate.

The Neurospora sulfite reductase possesses a hydroxylamine reductase activity as demonstrated by identical sedimentation behavior on sucrose density gradients and correlation of the activities in stored, dialyzed extracts of mutants or wild-type mycelia harvested at various stages of growth. As in bacteria (1, 12), there is a multiple genetic responsibility for sulfite/hydroxylamine reductase in Neurospora (14). Three genetically

![Fig. 4. Density gradient centrifugation of Neurospora extracts. Three-liter cultures of (wild-type) mycelia grown on minimal medium containing thiosulfate were harvested at 39 and 72 hours after inoculation, and extracts were prepared as described in the text, although without dialysis on Sephadex G-25. Density gradient centrifugation was performed on aliquots of each extract containing 3.9 mg of protein as described in the text. Enzyme activity units were expressed as follows: sulfite reductase, millimicromoles of \( \text{H}_2\text{S} \) per hour per 20 \( \mu \)l; TPNH-hydroxylamine reductase, \( \Delta E_{440} \) per 5 min per 20 \( \mu \)l for A and \( \Delta E_{440} \) per 5 min per 40 \( \mu \)l for B; catalase, \( \Delta E_{440} \) per min per 5 \( \mu \)l; and DPNH-hydroxylamine reductase, \( \Delta E_{440} \) per 10 min per 40 \( \mu \)l. Assay conditions were as described in the text. A (upper), 39-hour extract (total fractions = 73); B (lower), 72-hour extract (total fractions = 60).

![Fig. 5. Density gradient centrifugation of dialyzed, aged Neurospora extracts. Growth of mycelia was as described in Fig. 4. Extracts were prepared as described in the text. The preparations were dialyzed by passage through Sephadex G-25 and stored at \( -15^\circ \) for 100 hours. The thawed material (2.4 mg) was subjected to density gradient centrifugation as described in the text. Enzyme activity units were expressed as follows: TPNH-hydroxylamine reductase, \( \Delta E_{440} \) per 5 min per 40 \( \mu \)l, and catalase, \( \Delta E_{440} \) per min per 5 \( \mu \)l. Upper, 39-hour extract (total fractions = 62); lower, 72-hour extract (total fractions = 54).](http://www.jbc.org/)
distinct classes of mutants (cys-2, cys-4, and me-4) have been discovered which lack both activities. Additional studies on the enzymology of Neurospora sulfite reductase, to be presented in the accompanying paper (15), strongly support these conclusions.

The hydroxylamine reductase studied by Zucker and Nason (10) was prepared from mycelia which had been grown for 5 days in still culture. Leinweber and Monty (14) have demonstrated that the level of sulfite reductase decreases rapidly in still cultures after reaching a maximum at approximately 2 days of growth. In cultures grown with vigorous aeration, the rise and decline in activity (Fig. 2) seems to be accelerated considerably. From Figs. 1 and 4 it is evident that the decline in the unstable or Peak B hydroxylamine reductase activity is less rapid than that of the stable or Peak A activity. Thus, it is most probable that mycelia grown for 5 days would have very little sulfite reductase, and that the predominant hydroxylamine reductase activity would consist of the Peak B or C enzymes or both. Since the level of Peak C activity is much lower in all cases examined than the activities described by Zucker and Nason (10), it must be concluded that they were studying the Peak B activity. Further evidence supporting this conclusion is presented in the accompanying paper (15).

**SUMMARY**

Three species of reduced triphosphopyridine nucleotide-dependent hydroxylamine reductase activity were detected in extracts of Neurospora crassa. The species have been termed Peaks A, B, and C, in order of their relative sedimentation upon centrifugation in sucrose density gradients. The activities found in Peaks B and C will utilize either diphospho- or triphosphopyridine nucleotide for hydroxylamine reduction. The activity of Peak A is specific for TPNH. The activity of Peak B is quite unstable following dialysis.

Several lines of evidence indicate that Peak A hydroxylamine reductase is identical with sulfite reductase. (a) The sedimentation pattern of Peak A hydroxylamine reductase is coincident with that of Neurospora sulfite reductase. (b) The hydroxylamine reductase activity which is stable after dialysis and storage (Peaks A and C) correlates well with the level of sulfite reductase when the latter activity is allowed to vary as a function of age of mycelium at harvest. (c) “Stable” hydroxylamine reductase activity exhibits the same genetic dependence as does sulfite reductase. Three genetically distinct classes of mutants (cys-2, cys-4, and me-4) have been discovered which lack both activities.

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