Further Purification and Properties of Neurospora Nitrate Reductase*

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SUMMARY

*Neurospora crassa* (5297a) NADPH-nitrate reductase (NADPH: nitrate oxidoreductase, EC 1.6.6.2), a soluble, sulfhydryl-containing protein with FAD, a cytochrome *b* designated cytochrome *b*~55~ (N. crassa), and molybdenum as prosthetic groups, has been purified 500-fold by procedures including pH adjustment, ammonium sulfate fractionation of the resultant supernatant solution, phase separation to remove nucleic acids, diethylaminoethyl cellulose chromatography, hydroxylapatite chromatography, and, finally, Sephadex G-200 gel filtration. Other enzymatic activities associated with NADPH-nitrate reductase throughout the purification and maintaining a proportional relationship with it are NADPH-cytochrome *c* reductase, FADH~2~-nitrate reductase, and reduced methyl viologen-nitrate reductase. Polyacrylamide gel electrophoresis of the most highly purified enzyme preparations yielded a major buffalo black-staining zone containing the above enzymatic activities, a somewhat smaller zone, and several faint zones. In unstained gels, a red zone, which was apparently due to the presence of cytochrome *b*~55~, was visible at the position corresponding to the major buffalo black-staining zone.

By the use of sucrose density gradient centrifugation, a relative *s*~20w~ value of 8.0 for the nitrate reductase was found, and with Sephadex G-200 gel filtration techniques, a Stokes radius of 70 Å was determined. From the relationship, mol wt = 6nQNAS/(1 - cp), a molecular weight of 228,000 was calculated, assuming *v* = 0.725 cc per g.

All enzymatic activities associated with nitrate reductase are heat-labile but to varying degrees, with the NADPH-nitrate and -cytochrome *c* reductases being most sensitive, and FADH~2~- and reduced methyl viologen-nitrate reductase activities being progressively less so, in that order. The reduced methyl viologen-nitrate reductase activity showed a marked increase in activity as the NADPH activities declined.

The cytochrome *b*~55~ associated with nitrate reductase activity shows a typical cytochrome *b* type visible absorption spectrum at liquid nitrogen temperature and is a proto-

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zyme system, suggested the following electron transfer scheme establishing the sequence of several of the components of the enzyme system, together with the earlier work of Nicholas and Nason (6)

\[
\text{cytochrome } b_{56} \rightarrow \text{molybdenum} \rightarrow \text{NO}_3^-
\]

The similar flavin requirements for reduction of both nitrate and cytochrome \(c\), as well as the observation that reduced cytochrome \(c\) failed to reduce nitrate, account for the indicated position of cytochrome \(c\) in the above sequence.

The present paper reports on the details of the preparation of a more highly purified \textit{Neurospora} assimilatory nitrate reductase and the further characterization of the enzyme. Determination of the molecular weight of the enzyme, the properties of the several enzymatic activities associated with NADPH-nitrate reductase, and evidence suggesting the involvement of a second metal moiety are included. A preliminary account of a portion of this study has already appeared (7).

**Experimental Procedure**

**Culture Methods**

Growth of \textit{N. crassa} (wild type 5297a) was carried out at 25° on Fries basal medium (6) modified so that sodium nitrate was the sole nitrogen source. \textit{Neurospora} was subcultured and maintained by transfer and growth of conidia on Bacto-agar slants (5 ml) containing the same medium. The conidia from one slant grown for 4 to 7 days were suspended in 5 ml of sterile distilled water and used to inoculate 800 ml of liquid medium contained in a 2800-ml Fernbach flask. After 24 to 48 hours of growth on a reciprocating shaker to provide aeration, the contents of two or three Fernbach flasks were used to inoculate 100 liters of liquid medium in a New Brunswick Fermacell fermentor. Growth was allowed to continue for 15 to 18 hours under forced aeration of 5.5 to 6 cu ft of air per min. (Maximum growth under these conditions would be attained after approximately 24 hours.) Mycelia were collected by filtration of the culture through several layers of cheesecloth and washed with cold distilled water; the excess water was removed by filtration on Buchner funnels to give yields ranging from 700 to 1300 g, wet weight, per 100 liters of growth medium. The mycelia were stored at -15° until further use, usually after a period of 12 hours to 2 months.

**Substrates, Coenzymes, and Other Substances**

FAD (90 to 99% pure), FMN (100% pure), NADH (98% pure), 2-n-heptyl-4-hydroxyquinoline \(N\)-oxide, Tris as Trizma base, dextran 500, and cytochrome \(c\) (type III) were obtained from Sigma. Reduced cytochrome \(c\) was prepared by the method of Smith (8). NADPH (95% pure) was supplied by Sigma and Calbiochem. The Matheson Company, Inc., and the Endo Laboratories, Garden City, New York, provided 2-mecaptoethanol and the hydrolyzing enzyme, glusulase, respectively. Polyethylene glycol 6000, sodium nitrate, and other salts were reagent grade products of the J. T. Baker Chemical Company. Sodium dithionite (sodium hydrosulfite) and the reagents for the colorimetric assay of nitrite, sulfanilic acid and \(N\)-(1-naphthyl)ethylenediamine dihydrochloride, were obtained from Fisher Scientific. DEAE-cellulose (Whatman DE-22), provided by H. Reeve Angel, was prepared for chromatographic use according to their directions. Hydroxyxylapatite was purchased from the Clarkson Chemical Company as Hypatite-C, and Sephadex G-200 and dextran blue from Pharmacia. Methyl viologen was procured from K and K Laboratories, Brooklyn, and crystalline bovine serum albumin from Mann. The necessary reagents for preparation of polyacrylamide gels (9) were products of Eastman.

Buffer solutions were prepared by the method of Gomori (10) except that potassium instead of sodium phosphate salts were used and in some experiments, as indicated, phosphoric acid was used in place of hydrochloric acid to prepare Tris-P\(\_4\) buffer instead of Tris-HCl buffer.

**Spectral Measurements**

Difference spectra of enzyme preparations were measured at room temperature in cuvettes of 1-cm light path with the Cary model 14 recording spectrophotometer equipped with the 0 to 0.1, 0.1 to 0.2 optical density slide-wire. Spectra of preparations at liquid nitrogen temperatures were determined on the split beam spectrophotometer through the courtesy of Dr. Ronald W. Estabrook at the Johnson Foundation.

**Assay Procedures**

Protein—Protein was measured by a modification of the procedure of Lowry et al. (11), with crystalline bovine serum albumin as the standard, and by the measurement of absorbance at 250 \(\mu\)m and 260 \(\mu\)m as described by Layne (12).

Nitrite—The nitrite content of 0.5-ml reaction mixtures was determined colorimetrically by a modification of the diazo coupling procedure described by Nicholas and Nason (13), with 0.5 ml of 1%, w/v, sulfanilic acid in 20%, v/v, concentrated HCl followed by 0.5 ml of 0.12%, w/v, \(N\)-(1-naphthyl)ethylenediamine dihydrochloride and 0.5 ml of distilled water to give a final volume of 2.0 ml. After 10 min, the color intensity was determined with a Klett-Summerson colorimeter equipped with the No. 54 green filter. The presence of added reduced pyridine nucleotides at usual enzymatic assay levels did not interfere with color development to an extent where their removal would be warranted. However, the use of high NADPH and NADH concentrations during the determination of substrate saturation levels necessitated precipitation of the coenzymes by barium acetate before the measurement of nitrite could be undertaken. According to a standard curve, 10 Klett units represent 0.855 mmole of nitrite.

**Standard Enzyme Assays**

\textit{NADPH-Nitrate Reductase}—\textit{NADPH-nitrate reductase} was routinely determined and expressed in the same activity units as described previously (5).

However, in a few, indicated cases, activity was assayed by determining the rate of oxidation of NADPH by following the rate of decrease in absorbance at 340 \(\mu\)m in a reaction mixture
identical with that used for the assay based on nitrite formation, except that all volumes were doubled to give a 1-ml final volume. Enzyme preparations were added in amounts sufficient to give a decrease in absorbance of 0.04 to 0.2 per min, as measured either with the Gilford model 2000 absorbance recording photometer connected to a Beckman DU monochromator or with a Zeiss PMQ II spectrophotometer.

**FADH$_2$-Nitrate Reductase**—The reaction mixture for the determination of FADH$_2$-nitrate reductase consisted of 0.1 ml of 0.1 M NaNO$_3$, 0.1 ml of $10^{-2}$ M FAD, sufficient enzyme to result in the formation of 5 to 50 mmoles of nitrite, 0.03 ml of a freshly prepared solution of 8 mg of sodium dithionite per ml of 0.8% NaHCO$_3$ and 0.1 M phosphate buffer, pH 7.3, to give a final volume of 0.5 ml in a test tube, 13 × 100 mm. The reaction was started by the addition of the sodium dithionite and was allowed to proceed for 10 min. The assay mixture is essentially anaerobic under these conditions, owing to the presence of dithionite. The reaction was stopped by vigorous shaking of the test tube with an Adams Cycle-Mixer to destroy the dithionite, nitrite was determined by the procedure already mentioned, and the results were corrected for values obtained in identical reaction mixtures shaken at zero time. One unit of activity is defined as the formation of 1 mmmole of nitrite in 10 min.

**Reduced Methyl Viologen-Nitrate Reductase**—Essentially the same method of assay was used for reduced methyl viologen-nitrate reductase activity as for FADH$_2$-nitrate reductase, except that 0.1 ml of $10^{-2}$ M methyl viologen replaced the FAD and 0.02 ml rather than 0.03 ml of the sodium dithionite solution was added to initiate the reaction. The activity units are the same.

**NADPH-Cytochrome c Reductase**—FADH$_2$-cytochrome c reductase was determined as described previously (5), except that in some instances the assay was performed in 0.1 M phosphate buffer, pH 7.3, in order to provide conditions more similar to those used in the NADPH-nitrate reductase assay, thus allowing for a more valid comparison of the two activities. In these cases, 0.15 ml instead of 0.05 ml of 2% aqueous cytochrome c was necessary to ensure saturation of the enzyme. One unit of activity is defined as the reduction of 1 mmmole of cytochrome c per min. The extinction coefficient for cytochrome c used in calculations of activity units for this enzyme as well as for cytochrome c oxidase (below) was 2.1 × 10$^{4}$ cm$^2$ per mmmole (14).

**Cytochrome c Oxidase**—The assay mixture for cytochrome c oxidase activity consisted of 0.05 ml of a 5%, w/v, aqueous reduced cytochrome c solution (i.e. 4.1 × $10^{-2}$ M), sufficient enzyme preparation to give a decrease in absorbance at 550 m$m^2$ of 0.03 to 0.08 per min, and 0.1 m phosphate buffer, pH 7.3, to give a final volume of 1.0 ml. The reaction was initiated by the addition of the reduced cytochrome c and the rate of decrease in absorbance at 550 m$m^2$ was followed either with a Gilford recorder, model 2000, attached to a Beckman DU spectrophotometer, or with a Zeiss PMQ II spectrophotometer. One unit of activity is defined as the oxidation of 1 mmmole of reduced cytochrome c per min.

**RESULTS**

**Purification of NADPH-Nitrate Reductase**

Unless otherwise indicated, all purification steps were performed at 0-4°. In a typical purification, 2,500 g of frozen N. crassa mycelia were homogenized for 5 min in a commercial size (≈4-liter capacity) stainless steel Waring Blender, operated at low speed, containing a solution of 0.1 M phosphate buffer, pH 7.3, $10^{-4}$ M 2-mercaptoethanol, and $5 × 10^{-4}$ M EDTA (“preparation buffer”) and acid-washed glass pavement marking beads (3M Company). The homogenization was performed in four equal sized batches of 625 g of mycelia, 2.5 liters of preparation buffer, and 1,250 g of glass beads. The resulting crude homogenate was centrifuged for 20 min at 20,000 × g to yield a supernatant solution (crude extract or Fraction 1) and a pellet, which was discarded. The specific activity was 266 units per mg of protein; 10 similar preparations had specific activities that ranged from 174 to 636, with a mean of 353. The crude extract was adjusted to pH 5.0 to 5.2 by the dropwise addition, with constant stirring, of 300 ml of M citric acid. The mixture was centrifuged for 20 min at 20,000 × g and the precipitate was discarded; the supernatant solution (9,270 ml) was designated the pH 5 supernatant (Fraction 2).

Solid ammonium sulfate, in the proportion of 314 g per liter of preparation to give a final concentration of 50% saturation, was slowly added with constant stirring. Stirring was continued for 30 min and the mixture was then centrifuged for 20 min at 20,000 × g. The resulting supernatant solution was discarded and the precipitate was dissolved in 500 ml of preparation buffer to give a solution designated the first 50% ammonium sulfate precipitate (Fraction 3). (A recovery of somewhat more activity than that contained in the preceding pH 5 supernatant was consistently obtained, indicating either an inhibition of enzymatic activity in the pH 5 supernatant fraction or a reversible decrease due to dilution, since concentrating the enzyme tended to result in greater total activity.)

Nucleic acids were removed from the preparation by a phase separation technique performed in the following manner. To Fraction 3 were successively added, with stirring, 0.11 volume of a 20%, w/w, dextran 500 (dextran of average mol wt 500,000) solution and 0.3 volume of a 20%, w/w, polyethylene glycol 6,000 (polyethylene glycol of average mol wt 6,000) solution, followed by the slow addition of 166 g of sodium chloride, to yield a solution 4 M with respect to NaCl. The mixture was stirred for 2 hours and then centrifuged for 20 min at 1,500 × g, resulting in an upper phase containing the enzyme activity and a white, viscous lower phase containing nucleic acid. The upper phase was siphoned off, transferred to a dialysis sac, and dialyzed against 20 liters of a solution of 0.001 M phosphate buffer, pH 7.3, plus $10^{-4}$ M 2-mercaptoethanol and $5 × 10^{-4}$ M EDTA (“dialysis buffer”) for 2 hours. The dialysis bath was replaced twice by two fresh 20-liter batches of the same solution; dialysis was continued for 2 hours with each batch (dialyzed upper phase, Fraction 4).

Fraction 4 (1058 ml) was transferred to a DEAE-cellulose column (4.3 × 15.5 cm; column bed volume, 225 ml) previously equilibrated with a solution of dialysis buffer. The effluent contained approximately 30% of the total protein present in Fraction 4 but none of the enzymatic activity. The column was washed with 500 ml of dialysis buffer; the resulting effluent contained approximately 30% of the total protein of Fraction 4 but less than 1% of the total NADPH-nitrate reductase activity. The column was subsequently eluted with a linear gradient solution between 1 liter of dialysis buffer and 1 liter of 0.5 M phosphate buffer, pH 7.4, $10^{-4}$ M 2-mercaptoethanol, and $5 × 10^{-4}$ M EDTA. The fractions containing the greatest NADPH-nitrate reductase activity (representing that portion of the
gradient between 0.2 and 0.3 M phosphate) were pooled to give
the combined eluate (Fraction 5).

To 423 ml of Fraction 5 were added, with stirring, 132.5 g of
ammonium sulfate to give a final concentration of 50% satura-
tion. The solution was stirred for 1 hour and centrifuged for 20
min at 20,000 × g; the supernatant solution was discarded.
The precipitate was dissolved in 50 ml of preparation buffer (sec-
ond 50% ammonium sulfate precipitate, Fraction 6).

Fraction 6 (49.5 ml) was dialyzed for 6 hours against 800 ml of
preparation buffer containing 178 g of ammonium sulfate to give
a final concentration (enzyme plus dialysis bath) of 35% satura-
tion. The dialyzed enzyme was centrifuged for 20 min at 20,000
× g, the supernatant solution was discarded, and the supernatant solution
was dialyzed for 13 hours against a solution of 800 ml of prepara-
tion buffer containing 195 g of ammonium sulfate to give a final
concentration of 40% saturation. The 40% ammonium sulfate-
dialyzed enzyme was centrifuged for 20 min at 20,000 × g, the
supernatant solution was discarded, and the precipitate was
dissolved in a solution of 20 ml of preparation buffer (35 to 40% am-
onium sulfate dialysis precipitate, Fraction 7).

Fraction 7 (20 ml) was dialyzed for 1 hour against 1 liter of
dialysis buffer, the dialysis bath was replaced, and dialysis was
continued for an additional 14 hours. Dialyzed Fraction 7 was
added to a hydroxylapatite column (4.3 × 2.5 cm; column bed
volume, 3.5 ml), previously equilibrated with dialysis buffer,
and the column was then eluted successively with 70 ml of the dialysis buffer. The resulting effluents
had no activity. The column was then eluted successively with
70 ml each of 0.05, 0.20, 0.35, and 0.50 M phosphate buffer, pH
7.3, solutions containing 10−4 M 2-mercaptoethanol and 5 × 10−4 M EDTA to give, respectively, hydroxylapatite eluates 2, 3, 4,
and 5. Hydroxylapatite eluates 2 and 3 each had less than 6% of
the enzyme activity; hydroxylapatite eluates 3 and 4 had 37% and 30%, respectively. The specific activity of hydroxylapatite
eluate 4 (Fraction 8) was more than 4-fold greater than that of
eulate 3, and eluate 4 was therefore used for further purification of
the enzyme.

Fraction 8 (70 ml) was concentrated by dialysis against prepar-
ation buffer saturated with ammonium sulfate, thereby re-
moving about half the water and also precipitating the protein.
The saturated ammonium sulfate-dialyzed enzyme was centri-
guged for 20 min at 20,000 × g, the supernatant solution was
discarded, and the precipitate was dissolved in 1 ml of prepara-
tion buffer to yield a solution designated the concentrated hydro-
xyapatite eluate 4 (Fraction 9).

Fraction 9 was added to a Sephadex G-200 column (1.5 × 25
cm; column bed volume, 44 ml) previously equilibrated with preparation buffer. The column was eluted with the same solu-
tion and the contents of those tubes comprising peak NADPH-
nitrate reductase activity were pooled to give the fraction desig-
nated Sephadex G-200 eluates (Fraction 10). Fraction 10 had a
specific activity of 129,500 units per mg of protein, representing
a 487-fold purification over the crude extract. In 10 similar
purification runs, the specific activity ranged from 46,800 to
209,000 (average, 112,000), representing an average purification
of 317-fold. The pertinent data of the purification procedure are summarized in Table I.

Other Enzymatic Activities Associated with
NADPH-Nitrate Reductase

As shown in Table I (also see Fig. 2, below), several other enzymatic activities which appear to be associated with NADPH-
nitrate reductase, namely, flavin-dependent NADPH-cyto-
chrome c reductase, reduced FAD-nitrate reductase, and reduced
methyl viologen-nitrate reductase, can be followed during the
course of purification. After the first three steps of the purifica-
tion procedure, the above enzyme activities are retained in more
or less constant proportions, even after a 500-fold purification.
All show coincident elution profiles from DEAE-cellulose and
Sephadex gel filtration columns.

### Table I

**Summary of purification of NADPH-nitrate reductase and associated activities**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>NADPH-nitrate reductase</th>
<th>FADH2-nitrate reductase</th>
<th>Reduced methyl viologen-nitrate reductase</th>
<th>NADPH-cytochrome c reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>total activity</td>
<td>Specific activity</td>
<td>Recovery</td>
<td>total activity</td>
</tr>
<tr>
<td>1. Crude extract</td>
<td>9,750</td>
<td>75,000</td>
<td>19,900,000</td>
<td>260</td>
<td>100</td>
<td>2,840,000</td>
</tr>
<tr>
<td>2. pH 5 supernatant</td>
<td>0,270</td>
<td>27,800</td>
<td>11,700,000</td>
<td>120</td>
<td>50</td>
<td>1,640,000</td>
</tr>
<tr>
<td>3. 50% (NH4)2SO4 precipitate</td>
<td>1,060</td>
<td>9,850</td>
<td>13,700,000</td>
<td>1,208</td>
<td>60</td>
<td>2,240,000</td>
</tr>
<tr>
<td>4. dialyzed upper phase</td>
<td>1,060</td>
<td>9,850</td>
<td>13,700,000</td>
<td>1,208</td>
<td>60</td>
<td>2,240,000</td>
</tr>
<tr>
<td>5. Pooled DEAE eluates</td>
<td>425</td>
<td>2,050</td>
<td>7,410,000</td>
<td>3,670</td>
<td>37</td>
<td>1,545,000</td>
</tr>
<tr>
<td>6. 2nd 50% (NH4)2SO4 precipitate</td>
<td>50</td>
<td>1,340</td>
<td>8,750,000</td>
<td>6,530</td>
<td>44</td>
<td>1,275,000</td>
</tr>
<tr>
<td>7. 35-40% (NH4)2SO4 precipitate</td>
<td>20</td>
<td>293</td>
<td>5,600,000</td>
<td>19,250</td>
<td>28</td>
<td>882,000</td>
</tr>
<tr>
<td>8. Hydroxylapatite eluate 4</td>
<td>70</td>
<td>21.2</td>
<td>1,660,000</td>
<td>78,300</td>
<td>8.3</td>
<td>322,000</td>
</tr>
<tr>
<td>10. Pooled Sephadex G-200 eluates</td>
<td>8.5</td>
<td>7.65</td>
<td>991,000</td>
<td>120,500</td>
<td>5</td>
<td>193,500</td>
</tr>
</tbody>
</table>
Table II

**Cellular localization of NADPH-nitrate reductase**

Fresh mycelia (21 g) were suspended in 400 ml of digestion medium, containing 0.63 M sorbitol, citric acid (pH 5.8) - K_2HPO_4 buffer (0.1 M with respect to citric acid), 0.03 M 2-mercapto-ethylamine HCl, and 4 x 10^{-4} M EDTA. The crude snail gut enzyme, gluusulase (755,000 units), was added for enzymatic digestion of the cell walls, and the contents were gently shaken for 75 min at 30°C. The mixture was centrifuged for 5 min at 500 x g, and the collected hyphae were washed twice with 400 ml aliquots of cold 0.9 M sorbitol, resuspended in 40 ml of a chilled preparation medium (0.25 M sucrose, 0.005 M EDTA, pH 7.0, and 0.15% bovine serum albumin), and homogenized six times (six up and down cycles) in a TenBroeck glass homogenizer. The crude homogenate, which was diluted to 100 ml with the cold preparation medium and designated Fraction A, was centrifuged for 10 min at 1,500 x g and the precipitate (unbroken hyphae and large fragments) was discarded. The supernatant solution (Fraction B) was centrifuged for 30 min at 8,000 x g to give a supernatant solution (Fraction C) and a mitochondrial pellet (Fraction D). Fraction C was further centrifuged for 1 hour at 144,000 x g to yield a supernatant solution (Fraction E) and a pellet (Fraction F).

In order to determine whether NADPH-nitrate reductase resides in the cytoplasm of the hyphae or is associated with the mitochondria of *N. crassa* (becoming solubilized during the vigorous homogenization in the preparation of crude extracts), the cellular distribution of the enzyme among several fractions obtained from *Neurospora*, after partial enzymatic digestion of the cell walls according to the method of Greenawalt, Hall, and Wallis (15), was examined. Table II, which summarizes the cellular distribution of NADPH-nitrate reductase and cytochrome c oxidase activities, indicates that the soluble cytoplasmic fraction possessed virtually all the nitrate reductase activity (98%) but no cytochrome c oxidase activity. The latter activity was, in fact, almost entirely confined to the mitochondria (Fraction D) and mitochondrial fragments (represented in part by Fraction F). Electron micrographs of the mitochondrial pellet after glutaraldehyde fixation and osmium tetroxide staining showed that the preparation consists largely of intact mitochondria. In additional experiments (not shown) treatment for 1 hour in a Raytheon sonicator of washed *Neurospora* mitochondria, prepared by physical disruption instead of enzymatic degradation of the hyphae (15), did not result in the appearance of any NADPH-nitrate reductase activity.

**Purity of Enzyme**

Polyacrylamide gel electrophoresis at pH 8.3 (9) of the most highly purified NADPH-nitrate reductase preparation obtained thus far (specific activity, 209,000 units per mg of protein) followed by staining with buffalo black showed the presence of several proteins, as indicated in Fig. 1. In a similar but unstained gel, a red zone (probably cytochrome b_562) was visible at the position corresponding to the thickest and darkest buffalo black-staining zone of Fraction B.

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**Table II**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Total protein</th>
<th>NADPH-nitrate reductase</th>
<th>Cytochrome c oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg</td>
<td>Total activity</td>
<td>Recovery %</td>
</tr>
<tr>
<td>A. Crude homogenate...</td>
<td>100</td>
<td>1,200</td>
<td>15,400</td>
<td>100</td>
</tr>
<tr>
<td>B. 1,500 x g supernatant......</td>
<td>100</td>
<td>740</td>
<td>17,300</td>
<td>112</td>
</tr>
<tr>
<td>C. 8,000 x g supernatant......</td>
<td>100</td>
<td>600</td>
<td>20,600</td>
<td>134</td>
</tr>
<tr>
<td>D. 8,000 x g precipitate......</td>
<td>5</td>
<td>62</td>
<td>55</td>
<td>0.3</td>
</tr>
<tr>
<td>E. 144,000 x g supernatant...</td>
<td>96</td>
<td>547</td>
<td>22,100</td>
<td>143</td>
</tr>
<tr>
<td>F. 144,000 x g precipitate...</td>
<td>16</td>
<td>126</td>
<td>330</td>
<td>2.2</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Polyacrylamide gel electrophoresis at pH 8.3 of purified NADPH-nitrate reductase. The gels were prepared as described by Clark (9). A Sephadex G-200 eluate fraction was concentrated by pressure dialysis to a protein level of 3.3 mg per ml. To 0.10 ml of concentrated enzyme was added 0.05 ml of a 0.1%, w/v, bromphenol blue-10%, w/v, sucrose solution. A 75-μl (165 μg of protein, 34,500 activity units) portion of this mixture was layered onto the gel (~60 mm in length) shown at the left and a 50-μl (110 μg of protein, 25,000 activity units) sample onto the gel at the right. Electrophoresis was performed for approximately 3 hours at 400 volts and 4°C. The gels were then stained with buffalo black, and the unreacted dye was removed by washing with 7%, w/v, acetic acid.
The unstained gel was sliced with a razor blade into 28 sections of approximately 2 mm thickness. The sections were stored for several hours at -15°C in separate test tubes, each of which contained 0.5 ml of 0.1 M phosphate buffer, $10^{-8}$ M 2-mercapto-ethanol, and $5 \times 10^{-4}$ M EDTA, and then separately pulverized with a glass rod. Aliquots were withdrawn from each tube and assayed for the various activities associated with nitrate reductase. Fig. 2 shows the distribution of the various activities among the gel sections. The thickest and darkest staining zone (i.e. Section 7, which also contained the above mentioned visible red zone) displayed most of the NADPH-nitrate reductase activity, with only a small amount being present in Sections 8 through 10. FADH₂-nitrate reductase and NADPH-cytochrome c reductase activities showed a nearly identical pattern, whereas reduced methyl viologen-nitrate reductase coincided only in part with this pattern, tending toward an asymmetrical and wider distribution of activity.

Electrophoresis of the same enzyme preparation on polyacrylamide gel at pH 4.3 (with the electrodes reversed) produced a sample in which no discrete zones were visible after staining, resulting only in a faint smear throughout the middle portion of the gel.

**Preliminary Crystallization of NADPH-Nitrate Reductase**

NADPH-nitrate reductase was initially crystallized with the cooperation of Dr. William Jakoby by an ammonium sulfate procedure (16), with the use of a pooled Sephadex G-200 eluate enzyme preparation (Fraction 10, 21.3 mg of protein) with a specific activity of 63,000. Successive trituration of the enzyme (originally precipitated with a 50% saturated ammonium sulfate solution) with 45%, 42%, 40%, and 38% saturated ammonium sulfate in a solution of 0.05 M phosphate buffer, pH 7.3, $10^{-3}$ M 2-mercaptoethanol, and $5 \times 10^{-4}$ M EDTA at 0°C, followed by standing for 10 to 15 min at 0°C with occasional stirring, and centrifugation for 10 min at 8,000 × $g$, resulted in apparent crystallization of the enzyme within 1 hour in all four supernatant solutions provided they were allowed to stand at room temperature. The 42% saturated ammonium sulfate fraction, which gave the best yields, was allowed to stand for ~48 hours at room temperature and samples were withdrawn for photomicrography with the aid of a dark field condenser and an oil immersion lens (Fig. 3). The specific activity (69,100 units per mg) of the apparent crystals obtained at 42% ammonium sulfate saturation was not significantly different from that of the starting material (Fraction 10), with a recovery in total enzyme units of only ~5%. In none of the several crystallizations performed with the NADPH-nitrate reductase was there a significant increase in specific activity, and in most instances large activity losses occurred, since the enzyme is not stable to prolonged storage (more than 1 to 2 days) in ammonium sulfate solutions. In all cases examined, the apparent enzyme crystals possessed cytochrome $b_{59}$ ($N. crassa$) as determined spectrally.

**Behavior of NADPH-Nitrate Reductase upon Sucrose Density Gradient Centrifugation and Sephadex G-200 Gel Filtration**

Siegel and Monty (17) have reported that by the use of a sedimentation coefficient, $s$, determined by sucrose density

![Fig. 2. The distribution of the NADPH-nitrate reductase and associated activities after polyacrylamide gel electrophoresis at pH 8.3. A gel prepared identically to the gel depicted on the right in Fig. 1, with 110 µg of protein and 25,000 NADPH-nitrate reductase units, was not stained but was sliced into 28 roughly equal sections. The sections were then treated as described in the text and assayed for the presence of the various activities by the standard procedures. Of the 25,000 NADPH-nitrate reductase units applied to the gel, 2,294 units (~10%) were recovered from the gel slices. MVH → NO₃, reduced methyl viologen-nitrate reductase.](http://www.jbc.org/)
gradient centrifugation, and the Stokes radius, \(a\), reasonable estimates for the molecular weight of a macromolecule can be obtained. Table III summarizes the results and calculations for the sedimentation coefficients, or \(s_{20,w}\), value, of Neurospora NADPH-nitrate reductase obtained by sucrose density gradient centrifugation experiments according to the method of Martin and Ames (18), with yeast alcohol dehydrogenase and catalase as standards. The average sedimentation coefficient of the enzyme from the values given in Table III is 8.0.

Sephadex G-200 gel filtration chromatography as described by Ackers (19) was used to obtain an average value of the Stokes radius, \(a\), for the Neurospora NADPH-nitrate reductase of 98.3 Å (Table IV). The molecular weight by NADPH-nitrate reductase, based on these values and the assumed partial specific volume of 0.725, is about 228,000 with the upper limit of the hydrated molecule being greater than 1,000,000. A frictional ratio, \(f/f_o\), of 2.4 can also be calculated.

### Heat Lability of NADPH-Nitrate Reductase and Associated Activities

Fig. 4 shows the effects of exposure of the enzyme with time to a temperature of 49° on the several catalytic activities of the NADPH-nitrate reductase system. A rapid, concomitant loss of both NADPH-nitrate reductase activity and NADPH-cytochrome c reductase activity occurs, with less than 10% of either activity remaining after 3 min at 49°. In contrast, the reduced FADH2-nitrate reductase activity, after an initial decrease of 20%, remains essentially constant over a 30-min period at 49°, whereas the reduced methyl viologen-nitrate reductase shows a marked increase in activity, concomitant with the rapid decrease in the NADPH-nitrate and cytochrome c reductases. After 60 min at 55°, however, the reduced FADH2-nitrate reductase activity is lost, whereas the reduced methyl viologen activity is retained (not shown). The latter activity is finally destroyed after 100 min at 55° or 1 min at 68°.

### Low Temperature Absorption Spectrum of NADPH-Nitrate Reductase

The visible absorption spectrum of purified nitrate reductase at room temperature has already been reported and has been shown to be that of a \(b\) type cytochrome (b). The absolute absorption spectrum of a dithionite-reduced sample of highly purified NADPH-nitrate reductase at liquid nitrogen temperatures is shown in Fig. 5. An apparent shift of the spectrum toward shorter wave lengths relative to that obtained at room temperature was observed, with the \(\alpha\) peak now located at 552 µm (with a shoulder at 556 µm), the \(\beta\) peak at 524 µm (with a shoulder at 531 µm), and the Soret peak at 422 µm. Such shifts are expected at liquid nitrogen temperatures (21). The apparent band splitting of the \(\alpha\) peak (major absorption at 552 µm and a shoulder at 556 µm) is representative, and is commonly obtained in this region of the spectrum with cytochromes at low temperatures (21). Substrate (NADPH)-reduced samples of the same preparation showed essentially the same spectrum, although the intensity of absorption was somewhat less.

The linear relationship between cytochrome \(b\) content and nitrate reductase activity of various \(N.\ crassa\) enzyme fractions was previously indicated (5).

### Absorption Spectrum of Pyridine Hemochromogen of NADPH-Nitrate Reductase

The pyridine hemochromogen derivative of a Sephadex G-200 fraction (specific activity, 47,100) was prepared by the method of Paul, Theorell, and Akeson (22), as modified by Appleby and Morton (23). The spectrum of this derivative after reduction by sodium dithionite exhibits \(\alpha\), \(\beta\), and Soret peaks at approximately 554, 523, and 417 nm, respectively (Fig. 6), corresponding to those of a pyridine derivative of protoporphyrin IX.

### pH Optima

Fig. 7 shows the effect of \(pH\) and type of buffer on NADPH-nitrate reductase activity and NADPH-cytochrome \(c\) reductase activity. Both activities exhibit a broad \(pH\) optimum between 7 and 8. The difference in maximal NADPH-nitrate reductase activity between Tris-HCl and Tris-PO4 buffers can be attributed to the known phosphate requirement for maximal NADPH-nitrate reductase activity (4, 24). NADPH-cytochrome \(c\) reductase, however, does not display this phosphate requirement. Both reduced methyl viologen-nitrate reductase activity and reduced FAD-nitrate reductase activity also exhibit the same broad \(pH\) optimum between 7 and 9 (not shown). The reduced methyl viologen-nitrate reductase activity was definitely enhanced by phosphate—an enhancement similar to that observed...
Behavior of *N. crassa* NADPH-nitrate reductase upon Sephadex G-200 gel filtration

In Experiments 1 and 2, a Sephadex G-200 column of 2.5 × 30.0 cm was used. The void volume, \( V_v \), was 47 ml (as determined with P22 phage); the total volume, \( V_t \), 147 ml; and the gel matrix volume was calculated to be 3.0 ml, and therefore the inner volume, \( V_i \), was 97 ml, since \( V_i = V_t - V_v - V_g \). Mixtures of *N. crassa* NADPH-nitrate reductase, ferritin (cadmium-free, Nutritional Biochemicals), yeast alcohol dehydrogenase, catalase, and bovine serum albumin were applied in a volume of 1.0 ml to the column, and aliquots of approximately 1 ml were collected and examined for the various substances. The absorbance at 415 nm of the fractions was used to locate the position of the ferritin. Experiment 3 was performed in an identical fashion except that the Sephadex column used had dimensions of 2.5 × 37.5 cm and a \( V_v \) of 66 ml (as determined with dextran blue), a \( V_t \) of 183.5 ml, a \( V_g \) of 3.7 ml, and a \( V_i \) of 111.8 ml. The basic equation describing the operational parameters of a gel filtration or molecular sieve chromatography system as suggested by Gelotte (20) is

\[
K_d = \frac{V_v - V_g}{V_t - V_g - V_v} = \frac{V_v - V_g}{V_i}
\]

where \( V_v \) is the elution peak volume of a solute and \( K_d \) is the distribution coefficient of the solute (for example, a macromolecule such as a protein). Ackers (19), working with Sephadex G-200 columns, has related the distribution coefficient, \( K_d \), of a macromolecule to the Renkin equation for the restricted diffusion of a particle as follows

\[
K_d = \left(1 - \frac{a}{r}\right)^2 \left(1 - 2.104 \frac{a}{r} + 2.09 \left(\frac{a}{r}\right)^2 - 0.95 \left(\frac{a}{r}\right)^3\right)
\]

where \( a \) is the Stokes radius of the particle, and \( r \) the effective pore size of Sephadex G-200. The \( K_d \) values for each of the proteins shown in Table IV were calculated from experimental measurements of the corresponding \((V_v - V_g)/V_i\) ratios. The \( a/r \) values were then conveniently computed from the above Renkin equation by the use of the table published by Ackers (19) relating \( a/r \) ratios to the distribution coefficient. By the use of the known Stokes radius, \( a \), for each of the protein standards shown, an average \( r \) value of the Sephadex G-200 was obtained for a given experiment and used to calculate the Stokes radius of NADPH-nitrate reductase. In calculating molecular weight, \( \eta \) is the viscosity of the medium, \( N \) is Avogadro's number, \( \ell \) is the partial specific volume (assumed to be 0.725 cc per g), and \( p \) is the density of the medium.

### Table IV

<table>
<thead>
<tr>
<th>Substance and experiment No.</th>
<th>Distribution coefficient</th>
<th>( a/r ) ratio (Ackers (19))</th>
<th>Known ( a ) (Siegel and Monty (17))</th>
<th>Calculated ( a )</th>
<th>Calculated ( r )</th>
<th>Calculated mol wt (sphere)</th>
<th>Calculated mol wt from mol wt = ( 6\pi R\eta/(1 - \eta) )</th>
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</thead>
<tbody>
<tr>
<td>Ferritin</td>
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<td>243,000</td>
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<td><em>N. crassa</em> NADPH-nitrate reductase</td>
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<td>3</td>
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<td>0.26</td>
<td>52</td>
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<td>Yeast alcohol dehydrogenase</td>
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<td>Bovine serum albumin</td>
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</tr>
<tr>
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<td>0.18</td>
<td>35</td>
<td>194</td>
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</table>

For NADPH-nitrate reductase with the buffers shown in Fig. 7. In contrast, a comparison of the corresponding pH curves for the reduced FAD-nitrate reductase showed at best only a small enhancement of activity by phosphate. The Tris-PO₄ buffer is not as effective as PO₄²⁻ buffer, a situation which does not enhance the activity by phosphate. The Tris-PO₄ buffer had an inhibitory effect on activity. Furthermore, NADPH elicited an approximately 6-fold greater rate of nitrate reduction per unit of enzyme as compared with NADH, at saturating coenzyme concentrations. Although FAD gave only about a 35% greater enzymatic rate than FMN at their respective saturation levels, it had a \( K_m \) width of that of FMN.

**NADPH-Cytochrome c Reductase**—The rate of cytochrome c...

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**Substrate Affinities**

Table V presents a summary of the results obtained in experiments to determine the substrate affinities of the various enzymatic activities associated with *N. crassa* nitrate reductase. Several points deserve attention.

**NADPH-Nitrate Reductase**—NADPH is obviously the preferred electron donor, showing a 3-fold greater affinity for the enzyme. Furthermore, NADPH elicited an approximately 6-fold greater rate of nitrate reduction per unit of enzyme as compared with NADH, at saturating coenzyme concentrations. Although FAD gave only about a 35% greater enzymatic rate than FMN at their respective saturation levels, it had a \( K_m \) width of that of FMN.
Fig. 4. Effect of heating on NADPH-nitrate reductase and associated activities. A 0.9-ml portion of Fraction 10 (specific activity, 27,800 units per mg; 1.12 mg of protein per ml) contained in a test tube, 13 × 100 mm, was placed in a water bath at 49°. At the indicated times, 0.06-ml samples were withdrawn and pipetted into 0.54 ml of cold 0.1 M phosphate, pH 7.3. Each heat-treated sample was then assayed by the standard procedures for the various activities and the results were compared with those obtained with an unheated enzyme sample (2.2 μg of enzyme protein were used in the assays). MVH → NO₃, reduced methyl viologen-nitrate reductase.

Fig. 5. Absolute absorption spectrum of purified NADPH-nitrate reductase at liquid nitrogen temperatures. A 0.2-ml sample of a Sephadex G-200 eluate fraction (specific activity, 47,160) was diluted with 0.2 ml of glycerol and reduced by adding a few sodium dithionite crystals; its spectrum was determined in a split beam recording spectrophotometer with a mixture of 0.2 ml of 0.1 M phosphate buffer, pH 7.3, and 0.2 ml of glycerol in the reference beam.

Fig. 6. Absolute absorption spectrum of the pyridine hemochromogen derivative of NADPH-nitrate reductase. A 0.5-ml aliquot of a Sephadex G-200 eluate fraction (specific activity, 47,100) was treated with 0.2 ml of pyridine followed by 0.3 ml of 0.2 N KOH and then reduced by the addition of a few sodium dithionite crystals to form the reduced pyridine hemochromogen derivative of the cytochrome (22, 23). The spectrum of the derivative was measured against a reference cuvette containing 0.5 ml of distilled water, 0.2 ml of pyridine, and 0.3 ml of 0.2 N KOH.

reduction per unit of enzyme is also 6 times greater when NADPH is used as the electron donor instead of NADH, and NADPH again shows the greater affinity for the enzyme. The effect of saturating concentrations of flavin nucleotides on the rate of cytochrome c reduction in the NADPH-cytochrome c reductase assay was slightly higher (by about 10%) with FMN than with FAD. However, the Kₘ for FAD was about 1/9th of that for FMN.

The apparent Kₘ values for NADPH and FAD were found to be relatively independent of whether the NADPH-cytochrome c reductase assay was performed in 0.1 M phosphate buffer, pH 7.3, or 0.1 M Tris-HCl buffer, pH 8.0. In contrast, the cytochrome c saturation curves differed depending on the buffer used (Fig. 8). In both buffers, substrate inhibition was observed at cytochrome c concentrations greater than 30 × 10⁻⁴ M.

Reduced FAD-Nitrate Reductase—It is interesting to note that the Kₘ for reduced FAD here is 3 to 10,000 times greater than the Kₘ for FAD in those activities for which this flavin serves as an electron carrier.

Reduced Methyl Viologen-Nitrate Reductase—At a saturating concentration of 2 × 10⁻⁴ M reduced methyl viologen (or 10 nmoles per 0.5 ml reaction mixture), 18 nmoles of nitrite were formed during the 1-min reaction time. Therefore, methyl viologen is apparently acting as an electron carrier from sodium dithionite in the enzymatic reduction of nitrate.

Effect of Inhibitors on NADPH-Nitrate Reductase and Its Associated Activities

Inhibition by Sodium p-Hydroxymercuribenzoate—Nason and Evans (1) initially reported sodium p-hydroxymercuribenzoate to be an inhibitor of NADPH nitrate reductase. The effect of
FIG. 7. The effect of pH and type of buffer used on the rate of nitrate reduction (left) and cytochrome c reduction (right) with NADPH as the substrate. The assays were as described in "Experimental Procedure," except for the use of the indicated buffers at the different pH values. For the NADPH-nitrate reductase assays, 6.8 μg of enzyme (Sephadex G-200 eluate fraction) were used; 0.10 μg of enzyme were used in the NADPH-cytochrome c reductase assays.

**Table V**

Summary of substrate affinities of NADPH-nitrate reductase and associated activities

In all cases the $K_m$ values were derived from Lineweaver-Burk plots of the data.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>NADPH-nitrate reductase</th>
<th>NADPH-cytochrome c reductase</th>
<th>Reduced FAD-nitrate reductase</th>
<th>Reduced methyl viologen-nitrate reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electron donor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADPH</td>
<td>$6.2 \times 10^{-5}$</td>
<td>$1.9 \times 10^{-5}$</td>
<td>$1.4 \times 10^{-4}$</td>
<td>$6.3 \times 10^{-4}$</td>
</tr>
<tr>
<td>NADH</td>
<td>$2 \times 10^{-4}$</td>
<td>$1.5 \times 10^{-5}$</td>
<td>$1.2 \times 10^{-4}$</td>
<td>$0.6 \times 10^{-4}$</td>
</tr>
<tr>
<td>FADHz</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduced methyl viologen</td>
<td>$2 \times 10^{-4}$</td>
<td>$1.1 \times 10^{-4}$</td>
<td>$3.4 \times 10^{-4}$</td>
<td></td>
</tr>
<tr>
<td>Electron acceptor</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrate</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Cytochrome c</td>
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<td></td>
</tr>
<tr>
<td>Phosphate</td>
<td></td>
<td>$4 \times 10^{-8}$</td>
<td>$3.4 \times 10^{-6}$</td>
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</tr>
<tr>
<td>Tris</td>
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<td>$1.4 \times 10^{-5}$</td>
<td>$3.4 \times 10^{-6}$</td>
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<tr>
<td>Electron carrier</td>
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<tr>
<td>FAD</td>
<td>$1.7 \times 10^{-3}$</td>
<td>$4 \times 10^{-8}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FMN</td>
<td>$3.8 \times 10^{-4}$</td>
<td>$4 \times 10^{-8}$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Increasing concentrations of sodium p-hydroxymercuribenzoate on the several enzymatic activities of NADPH-nitrate reductase is shown in Table VI. Of the activities examined, the two which use NADPH (i.e. NADPH-nitrate reductase and NADPH-cytochrome c reductase) are most sensitive to the inhibitor. Such behavior indicates involvement of a sulphydryl group on the enzyme in the initial portion of the electron transfer sequence. FADHz-nitrate reductase and reduced methyl viologen-nitrate reductase are both considerably less sensitive to p-hydroxymercuribenzoate. Significant inhibition of the reduced methyl viologen- and reduced FAD-nitrate reductase systems is attained only at concentrations where the function of p-hydroxymercuribenzoate as a specific sulphydryl group poison is questionable.

Of some interest is the significant stimulation of reduced methyl viologen-nitrate reductase activity in the range of final p-hydroxymercuribenzoate concentrations between $10^{-5}$ and $10^{-4}$ M, a phenomenon also produced by heat treatment of the enzyme (Fig. 4).

Inhibition by Metal-binding Agents—Table VII shows the degree of inhibition of the various activities caused by several metal-binding agents, KCN, NaN₃, and thiourea, each resulting in significant inhibition of those activities for which nitrate served as the electron acceptor. The data support the notion that a metal, already indicated to be molybdenum by earlier studies (2, 3), functions in the terminal portion of the electron transfer scheme. The reason for the decreased sensitivity of
2880 NADPH-Nitrate Reductase

0.1 protein (Sephadex G-200 eluate fraction) was used.

mental Procedure." i.e. in 0.1 performed under the two sets of conditions described in "Experimental Procedure," i.e. in 0.1 Tris-HCl buffer, pH 7.3, and in 0.1 M phosphate buffer, pH 8.0, the only variable being the changing cytochrome c concentration. For each assay mixture, 0.068 μg of protein (Sephadex G-200 eluate fraction) was used.

FADH2-nitrate reductase to these agents as compared with NADPH- and methyl viologen-nitrate reductases is unclear, and is possibly some nonenzymatic interaction between FAD and inhibitor. NADPH-cytochrome c reductase was unaffected by cyanide, azide, or thiourea. The ability of KCN, NaN3, and thiourea to inhibit NADPH-nitrate reductase was first reported by Nason and Evans (1), who also showed o-phenanthroline and S-hydroxyquinoline to be inhibitors of the enzyme. As seen in Table VII, o-phenanthroline and S-hydroxyquinoline at high concentrations caused significant inhibition of both NADPH-nitrate reductase and NADPH-cytochrome c reductase, but had no effect on the FADH2- and reduced methyl viologen-nitrate reductases, which are quite sensitive to cyanide, azide, and thiourea. The results suggest a possible involvement of a second metal moiety (perhaps nonheme iron), acting in the initial portion of the electron transfer pathway.

Table VII also illustrates the failure of 2-n-heptyl-4-hydroxyquinoline N-oxide to inhibit NADPH nitrate or cytochrome c reductase at 100 times the concentration needed to block the terminal respiratory pathway in mammalian systems. Moreover, it did not affect the appearance of the typical reduced cytochrome b557 spectrum upon subsequent addition of NADPH and FAD to the enzyme fraction. The inhibitor has been reported to act at the level of cytochrome b, and does inhibit the respiratory nitrate reductase pathway of E. coli, presumably at the cytochrome b5 site (25). Other b type cytochromes, in addition to the Neurospora cytochrome b56 discussed here, are also insensitive.

Effect of Nitrogen Compounds on NADPH-Nitrate Reductase—The following compounds, at a final concentration of 10^-5 M, which were tested with a view to their action in a negative feedback phenomenon, had no effect on NADPH-nitrate reductase activity as measured by following the rate of NADPH oxidation at 340 mp: ammonium chloride, urea, glycine, DL-alanine, DL-aspartic acid, L-asparagine, L-glutamic acid, L-glutamine, L-valine, L-histidine, and L-phenylalanine. Hydrazine caused an inhibition of 40% at 10^-3 M, and hydroxylamine hydrochloride inhibited 88% at this concentration. Interestingly enough, nitrite appeared to serve as an electron acceptor in this system, as determined by the rate of NADPH oxidation, although less effectively than nitrate. The oxidation of NADPH by nitrite concentrations from 10^-5 to 10^-3 M ranged from about 15% to a maximal value of 45% of that attained with nitrate. Also, oxidation of NADPH in the presence of both NO3^- and NO2^- is not equal to the sum of NADPH oxidation by NO3^- and NO2^- individually, suggesting that these acceptors might be reduced at the same enzymatic site. Nitrite at concentrations found in the usual NADPH-nitrate reductase assay for nitrite formation (10^-5 to 5 x 10^-5 M) had little or no effect on NADPH oxidation by nitrate.

Table VI

\[ \text{Activity remaining} \]

\begin{tabular}{|c|c|c|c|c|}
\hline
\text{p-Hydroxymercuribenzoate} & \text{NADPH-nitrate reductase} & \text{NADPH-cytochrome c reductase} & \text{Reduced methyl viologen-nitrate reductase} & \text{Reduced FAD-nitrate reductase} \\
\hline
\text{m} & \text{% original activity} & \text{Original activity} & \text{Original activity} & \text{Original activity} \\
\hline
0 & 100 & 100 & 100 & 100 \\
10^-3 & 0 & 27 & 31 & 31 \\
2 x 10^-4 & 0 & 124 & 85 & 85 \\
10^-4 & 0 & 153 & 91 & 91 \\
5 x 10^-3 & 0 & 0 & 206 & 106 \\
2 x 10^-3 & 0 & 0 & 211 & 96 \\
10^-3 & 0 & 0 & 0 & 0 \\
8 x 10^-4 & 0 & 0 & 0 & 0 \\
6 x 10^-4 & 0 & 0 & 0 & 0 \\
5 x 10^-4 & 0 & 15 & 0 & 0 \\
4 x 10^-4 & 34 & 34 & 34 & 34 \\
3 x 10^-4 & 42 & 42 & 42 & 42 \\
2 x 10^-4 & 49 & 81 & 105 & 105 \\
10^-6 & 63 & 7 & 89 & 89 \\
8 x 10^-7 & 11 & 11 & 11 & 11 \\
7 x 10^-7 & 9 & 9 & 9 & 9 \\
6 x 10^-7 & 36 & 36 & 36 & 36 \\
5 x 10^-7 & 44 & 44 & 44 & 44 \\
4 x 10^-7 & 85 & 85 & 85 & 85 \\
3 x 10^-7 & 91 & 91 & 91 & 91 \\
2 x 10^-7 & 70 & 88 & 100 & 100 \\
\hline
\end{tabular}

FIG. 8. The effect of the concentration of cytochrome c on NADPH-cytochrome c reductase activity. The assays were performed under the two sets of conditions described in "Experimental Procedure," i.e. in 0.1 M Tris-HCl buffer, pH 7.3, and in 0.1 M phosphate buffer, pH 8.0, the only variable being the changing cytochrome c concentration. For each assay mixture, 0.068 μg of protein (Sephadex G-200 eluate fraction) was used.
The enzymatic activities were measured by the standard procedures, except for the inclusion of the indicated inhibitors at the concentrations shown. The data are expressed as percentage of the uninhibited rate. In cases in which the inhibitor was dissolved in ethanol, control experiments with equivalent amounts of ethanol provided data for the uninhibited rate. Various Sephadex G-200 eluate fractions were used: 6.8 to 60.8 μg of enzyme protein were added in the NADPH-, FADH2-, and reduced methyl viologen-nitrate reductase assays; 0.10 to 1.40 μg were added in the assays of NADPH-cytochrome c reductase.

The following experimental evidence reported here supports the viewpoint that the electron transport system responsible for assimilatory nitrate reduction in *N. crassa* is a discrete enzyme system represented under physiological conditions by a single physical entity, made up, perhaps, of several tightly bound polypeptide chains: (a) all activities known to be associated with nitrate reductase, i.e. NADPH-nitrate reductase, NADPH-cytochrome c reductase, FADH2-nitrate reductase, and reduced methyl viologen-nitrate reductase, show coincident elution profiles from DEAE-cellulose and Sephadex gel filtration columns, (b) all activities keep a somewhat constant proportion even after a 500-fold purification (Table I), and (c) all activities are associated with a particular protein zone after polyacrylamide gel electrophoresis (Fig. 2). Previous studies (4) have shown that NADPH-nitrate reductase and NADPH-cytochrome c reductase are induced concomitantly in mycelia presented with nitrate.

In addition, a plausible explanation can be given for the observed differential effects of the 49 °C temperature treatment on the several catalytic activities of the enzyme that is consistent with the notion that nitrate reductase is a single system. Presumably, the first step of the over-all catalysis, involving NADPH, is carried out by a portion of the enzyme system possessing a greater degree of heat lability, due perhaps to a corresponding sensitivity of the tertiary structure in this portion of the complex. Concomitant with this assumed loss of tertiary structure, it is possible that the accessibility of the substrate, reduced methyl viologen, to its enzymatic site is increased, thus accounting for the enhanced level of activity. According to this reasoning, reduced FAD does not suffer from a limited accessibility to its site of oxidation, since no increase is seen. Further temperature increases result in an over-all deterioration of enzyme complex structure, with the resultant loss of all activity. A comparable explanation can be invoked to account for similar pattern resulting from p-hydroxymercuribenzoate treatment (Table VI). Any alternate hypothesis explaining these results on the assumption of differential heat (or p-hydroxymercuribenzoate) lability of physically unassociated but coordinately acting enzymes in free solution would have to attribute the increase in reduced methyl viologen-nitrate reductase that accompanies the decrease in NADPH activities to coincidence (Fig. 4).

The inhibitory pattern exhibited by sodium p-hydroxymercuribenzoate on the activities associated with the nitrate reductase system (Table VI) indicates the involvement of one or more relatively accessible sulfhydryl groups in the initial portion of the electron transfer sequence (functioning perhaps in the binding of NADPH) and probably less accessible SH groups at one or more sites between FAD and nitrate. The alternate possibility that a sulfhydryl group (or groups) may be serving as acceptors of electrons from NADPH is less likely in view of earlier findings (5) that sulfhydryl reagents, i.e. cysteine and glutathione, cannot serve as electron donors for nitrate reductase.

Indications that cytochrome *b* _551* (N. crassa) is involved in the electron transfer sequence of nitrate reductase, presented earlier (5), are further supported by the presence of a visible red zone after polyacrylamide gel electrophoresis of purified enzyme in that section of the gel possessing NADPH-nitrate reductase and associated activities (Fig. 4).

Several measurements performed on large quantities of partially purified preparations (specific activity, 6,000 to 13,000) indicated the presence of 1 to 2 moles of molybdenum per mole of NADPH-nitrate reductase (assuming NADPH-nitrate reductase to be the only molybdenum-containing protein present). The results of experiments to determine the effect of metal-binding agents on NADPH-nitrate reductase activity essentially confirmed previous observations. However, in addition, it was found that the NADPH-cytochrome c reductase activity of the nitrate reductase system was inhibited by o-phenanthroline and 8-hydroxyquinoline but not by cyanide, azide, or thiourea, whereas the inverse situation was obtained with FADH2 and reduced methyl viologen-nitrate reductase activities (Table VII). Moreover, NADPH-nitrate reductase was also inhibited by

### Table VII

<table>
<thead>
<tr>
<th>Inhibitor and final concentration (mM)</th>
<th>NADPH-nitrate reductase inhibition</th>
<th>NADPH-cytochrome c reductase inhibition</th>
<th>Reduced methyl viologen-nitrate reductase inhibition</th>
<th>Reduced FADH2-nitrate reductase inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>o-Phenanthroline</td>
<td>10^-3: 52% 58% 0% 0%</td>
<td>10^-4: 0% 0%</td>
<td>10^-5: 71% 0% 51% 28%</td>
<td>10^-5: 0% 0% 24% 0% 4% 0%</td>
</tr>
<tr>
<td>5 × 10^-3</td>
<td>10^-3: 52% 58% 0% 0%</td>
<td>10^-4: 0% 0%</td>
<td>10^-5: 71% 0% 51% 28%</td>
<td>10^-5: 0% 0% 24% 0% 4% 0%</td>
</tr>
<tr>
<td>10^-3</td>
<td>10^-3: 52% 58% 0% 0%</td>
<td>10^-4: 0% 0%</td>
<td>10^-5: 71% 0% 51% 28%</td>
<td>10^-5: 0% 0% 24% 0% 4% 0%</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>10^-3: 35% 0% 0% 0%</td>
<td>10^-3: 35% 0% 0%</td>
<td>10^-3: 35% 0% 0% 0%</td>
<td>10^-3: 35% 0% 0% 0% 24% 0%</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>10^-3: 99% 0% 99% 91%</td>
<td>10^-3: 99% 0% 99% 91%</td>
<td>10^-3: 99% 0% 99% 91%</td>
<td>10^-3: 99% 0% 99% 91% 24% 0%</td>
</tr>
<tr>
<td>Thiourea</td>
<td>10^-3: 47% 0% 51% 34%</td>
<td>10^-3: 47% 0% 51% 34%</td>
<td>10^-3: 47% 0% 51% 34%</td>
<td>10^-3: 47% 0% 51% 34% 24% 0%</td>
</tr>
<tr>
<td>2,4-Heptyl-4-hydroxyquinoline N-oxide</td>
<td>10^-2: 24% 0% 4% 0%</td>
<td>10^-2: 24% 0% 4% 0%</td>
<td>10^-2: 24% 0% 4% 0%</td>
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</tr>
</tbody>
</table>

**DISCUSSION**

The following experimental evidence reported here supports the viewpoint that the electron transport system responsible for assimilatory nitrate reduction in *N. crassa* is a discrete enzyme system represented under physiological conditions by a single physical entity, made up, perhaps, of several tightly bound polypeptide chains: (a) all activities known to be associated with nitrate reductase, i.e. NADPH-nitrate reductase, NADPH-cytochrome c reductase, FADH2-nitrate reductase, and reduced methyl viologen-nitrate reductase, show coincident elution profiles from DEAE-cellulose and Sephadex gel filtration columns, (b) all activities keep a somewhat constant proportion even after a 500-fold purification (Table I), and (c) all activities are associated with a particular protein zone after polyacrylamide gel electrophoresis (Fig. 2). Previous studies (4) have shown that NADPH-nitrate reductase and NADPH-cytochrome c reductase are induced concomitantly in mycelia presented with nitrate.

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o-phenanthroline and 8-hydroxyquinoline. The above observations implicate, in addition to the already established occurrence of molybdenum in the terminal portion of the electron transport process, the existence of a second metal component, susceptible to metal-binding agents, earlier in the nitrate reductase chain, perhaps near the flavin site. Nonheme iron has been implicated as a constituent of other nitrate reductases at the flavin site (26).

Perhaps near the flavin site. Nonheme iron has been implicated to metal-binding agents, earlier in the nitrate reductase chain, in addition to the already established occurrence of o-phenanthroline and 8-hydroxyquinoline. The above observations implicate, in addition to the already established occurrence of molybdenum in the terminal portion of the electron transport process, the existence of a second metal component, susceptible to metal-binding agents, earlier in the nitrate reductase chain, perhaps near the flavin site. Nonheme iron has been implicated as a constituent of other nitrate reductases at the flavin site (26).

It seems unlikely that o-phenanthroline or 8-hydroxyquinoline might be acting on the iron atom in the porphyrin of cytochrome b₅₆₅, since heme-bound metal atoms are insensitive to the action of chelating agents. However, since the concentrations of these two metal-binding agents necessary to produce significant inhibition are quite high, their role must be interpreted with caution. A tenous assumption in this rationale is that an exogenously added reduced cofactor, such as FADH₂, functions in an electron transfer role at the same site as under more nearly physiological conditions. At least one fact which argues against this assumption is the observed 3000-fold difference in Kₘ values between FAD and FADH₂.

The above data are consistent with the following electron transfer scheme, which is a modification of that originally proposed (6).

\[
\text{NADPH} \rightarrow \text{FAD} \rightarrow \text{cytochrome c} \rightarrow \text{cytochrome b₅₆₅} \rightarrow \text{molybdenum} \rightarrow \text{NO₃⁻}
\]

\[(\text{metal?})\]

Reduced methyl viologen serves as an electron donor prior to the molybdenum site, either before or after cytochrome b₅₆₅, but its precise locus of action has not been ascertained. It will neither accept electrons from NADPH in the presence or absence of FAD, nor replace FAD in NADPH oxidation by nitrate. The molecular weight of the nitrate reductase complex has been estimated to be approximately 228,000, a size which could easily accommodate the model of several intimately linked proteins or polypeptides, each responsible for a single step in the over-all electron transfer sequence of NADPH-nitrate reductase. Finally, the present finding that Neurospora assimilatory nitrate reductase appears to be a soluble component of the cytoplasm, which is not associated with mitochondria, suggests a major criterion for distinguishing it from the dissimilatory type of nitrate reductase.

The results obtained for the effects of various nitrogen compounds suggest there is no apparent control of the nitrate reductase system through a negative feedback mechanism at the enzymatic level. The inhibitory effect of hydrazine and hydroxylamine can best be ascribed to their metal-binding properties at high concentrations.

**REFERENCES**

Further Purification and Properties of *Neurospora* Nitrate Reductase
Reginald H. Garrett and Alvin Nason


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