Characterization of a Nitrate Reductase from the Chemoautotroph *Nitrobacter agilis*

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*Nitrobacter agilis* was isolated and named by Winogradsky (1, 2) in 1890 and was reported to be an obligate chemoautotroph, the primary energy source of which is derived from the oxidation of nitrate to nitrite according to the equation

\[ \text{NO}_3^- + \frac{1}{2} \text{O}_2 \rightarrow \text{NO}_2^- + 17.8 \text{ kcal} \]  

(3)

The energy thus liberated is used in part for the assimilation of carbon dioxide which serves as the sole carbon source for growth.

Studies of the biochemistry of *Nitrobacter agilis* have been mainly limited by the difficulty in obtaining large yields of cells, as has been the case with many of the chemoautotrophs. There has consequently been a relatively slow advancement of biochemical research concerning the mechanism of nitrification as well as that of other metabolic pathways in these organisms. Aleem and Alexander (4) have recently devised culture methods for growing the organism in approvable yields, thus opening the way for studies with cell-free preparations of the bacteria. Most of the subsequent investigations with *Nitrobacter* have been mainly concerned with the cell-free oxidation of nitrate to nitrite. Aleem and Nason (5) demonstrated that the nitrite-oxidizing activity of these cell-free preparations resides solely in a cytochrome-containing particle designated as nitrite oxidase. Spectral evidence suggested that nitrite oxidation involved the enzymatic transfer of electrons from nitrite to molecular oxygen via cytochrome c and cytochrome a1 according to the following sequence.

\[ \text{NO}_2^- \rightarrow \text{cytochrome c} \rightarrow \text{cytochrome a1} \rightarrow \text{O}_2 \]

This specific enzymatic oxidation of nitrite by molecular oxygen in *Nitrobacter agilis* was then shown (6) to be coupled to the formation of high energy phosphate bonds. Aleem and Nason (7) have subsequently found that reduced diphosphopyridine nucleotide and adenosine triphosphate stimulate the incorporation of carbon dioxide into cell-free extracts of this nitrifying bacterium. Spectral results obtained in this investigation were discussed by Alexander (4). When the stationary phase of growth was reached (usually at 1 to 2 weeks), the cultures were cooled overnight at 0–4°C and harvested by centrifugation in a refrigerated Sharples ultracentrifuge at 50,000 r.p.m. and at a flow rate of 10 to 20 liters per hour. The collected precipitate was washed by centrifugation four or five times in 0.1 M Tris buffer, pH 7.5, suspended in Tris buffer at a concentration of 1 g, wet weight, per 5 ml of buffer, and stored at -15°C. The yield obtained from each culture was 3 to 4 g of cells, wet weight.

**Experimental Procedure**

**Culture Methods**—Cells of *Nitrobacter agilis* (ATCC 9482) were grown in a clear inorganic culture solution as described by Aleem and Alexander (4). When the stationary phase of growth was reached (usually at 1 to 2 weeks), the cultures were cooled overnight at 0–4°C and harvested by centrifugation in a refrigerated Sharples ultracentrifuge at 50,000 r.p.m. and at a flow rate of 10 to 20 liters per hour. The collected precipitate was washed by centrifugation four or five times in 0.1 M Tris buffer, pH 7.5, suspended in Tris buffer at a concentration of 1 g, wet weight, per 5 ml of buffer, and stored at -15°C. The yield obtained from each culture was 3 to 4 g of cells, wet weight.

**Substrates, Cofactors, and Other Substances**—Digitonin (A grade) was obtained from the California Corporation for Biochemical Research, and 2% solutions were prepared by dissolving in 5 N NaOH and neutralization with 5 N HCl (11). Reduced horse heart cytochrome c (Sigma Chemical Company) was prepared by treatment of a 5% solution of either type II (65 to 72% pure) or type III (100% pure) with palladium asbestos and hydrogen gas (12). Crystalline bovine albumin and Triton X-100 were provided by Mann Research Laboratories and Rohm and Haas (Philadelphia), respectively. Solutions of 2,6-dichlorophenolindophenol (Eastman Organic Chemicals) were reduced with asbestos and hydrogen gas essentially as described by Smith and Stotz (13). Sulfanilic acid and N-[1-naphthyl]ethylenediamine dihydrochloride were obtained from the Baker Chemical Company and the Fisher Scientific Company.

**Spectral Measurements**—Difference spectra of cell-free preparations at room temperature were obtained with the Cary model 14 recording spectrophotometer, with the use of the sensitive slide wire (0 to 0.2 optical density unit) and cuvettes of 1-cm light path. Spectra of preparations at liquid nitrogen temperatures were recorded with a split beam spectrophotometer.†

**Sedimentation Studies**—Sedimentation coefficients were determined in a Beckman model E analytical ultracentrifuge with schlieren optics and standard and wedge cells with Krylon-coated

† We are indebted to Dr. Ronald W. Estabrook at the Johnson Foundation for the low temperature spectral analyses.
aluminum 2° centerpieces. The temperature of the rotor was maintained at 5°, and all experiments were conducted at 59,780 r.p.m.

**Analytical Methods**—The nitrite content of the 1-ml reaction mixture was determined colorimetrically by the addition of 0.5 ml of 1% (w/v) sulfanilic acid in 2 N HCl followed by 0.5 ml of 0.12% (w/v) N-(naphthyl)ethylenediamine dihydrochloride. Distilled water was added to a final volume of 2.4 ml. After 10 minutes the resulting red color was measured on the Klett-Summerson colorimeter with a No. 54 green filter (500 to 560 mp). Cytochrome c did not interfere with this determination. According to a standard curve, 10 Klett units represent 9.52 × 10^-4 μmole of nitrite.

Cytochrome c was determined as follows. For determination of the total concentration of cytochrome c (oxidized plus reduced), in any given solution, the solution was first fully reduced by the addition of a few crystals of dithionite. Its absorbance was then measured in a Beckman DU spectrophotometer at 550 mp, and the cytochrome c concentration was calculated on the basis of an extinction coefficient of 2.99 × 10^8 m^-1 cm^-1 at 550 mp for reduced cytochrome c (13). The percentage of reduced cytochrome c in a given solution was obtained by measuring absorbance at 550 mp of (a) the sample, (b) the sample fully reduced with dithionite, and (c) the sample fully oxidized with potassium ferricyanide, and then applying the equation

% Reduced = \[ \frac{A_{sample} - A_{oxidized}}{A_{reduced} - A_{oxidized}} \times 100 \]

The amount of reduced cytochrome c which was oxidized during a reaction was obtained by applying the extinction coefficient of 2.1 × 10^8 m^-1 cm^-1 (15) to the observed decrease in optical density at 550 mp.

Protein was determined by the Lowry method as described by Layne (16) with bovine serum albumin as a standard.

**Standard Assay for Nitrate Reductase and Cytochrome Oxidase**—The reaction mixture for the determination of nitrate reductase activity, in a test tube 13 × 100 mm, consisted of 0.5 ml of 0.1 M acetate buffer (pH 6.0), 0.1 ml of 0.1 M KNO₃, 0.1 ml of the suitably diluted enzyme preparation, 0.03 ml of a 5% solution of reduced cytochrome c (final concentration of reduced cytochrome c, 3 to 6 × 10^-5 M), and distilled water to a final volume of 1.0 ml. The reaction was started by the addition of either enzyme or reduced cytochrome c and was allowed to proceed aerobically at room temperature for 5 minutes. The addition of sulfanilic acid terminated the reaction, and the contents of each tube were analyzed for the appearance of nitrite. The Klett readings thus obtained were corrected with an identical reaction mixture stopped at zero time. One unit of activity is defined as 1 Klett unit.

In some cases nitrate reductase activity was also measured by the rate of oxidation of reduced cytochrome c as determined by the decrease in absorbance at 550 mp measured at 30-second intervals in a Beckman DU spectrophotometer. These readings were corrected with the corresponding decrease in absorbance which occurred without added enzyme.

An identical reaction mixture was used for the determination of cytochrome oxidase activity except that nitrate was omitted from the cuvette. Activity was determined by the decrease in absorbance at 550 mp at 30-second intervals corrected by the nonenzymatic decrease in absorbance.
Nitrate Reductase from Nitrobacter agilis

Whole cells (2 g, wet weight, in 10 ml of 0.1 M Tris, pH 7.5)

- Sonic oscillation, 40 minutes
- Sonic extract
- Centrifuge at 3,000 X g, 20 minutes

Pellet (20%)
Discard

Supernatant (80%) (Fraction I)

- Centrifuge at 144,000 X g, 2 hours

Pellet (90%) (Fraction II)
Resuspend in 0.5 original volume in 0.1 M acetate buffer, pH 6.0

Homogenize with equal volume of 2% digitonin, 30 minutes

Digitonin suspension (150%) (Fraction III)

- Centrifuge at 144,000 X g, 30 minutes

Pellet (10%)
Discard

Digitonin supernatant (90%) (Fraction IV)

- Negative adsorption on calcium phosphate gel
- Discard

Supernatant (90%) (Fraction V)

- Positive adsorption on calcium phosphate gel
- Elute in 0.5 original volume with pyrophosphate buffer, pH 6.5

Eluate (50%) (Fraction VI)

- Treat with 65% ammonium sulfate, 2 to 3 days
- Centrifuge at 17,000 X g, 20 minutes

Pellet (85%) (Fraction VIII)
Resuspend in 0.1 M acetate, pH 6.0

Supernatant (15%) (Fraction VII)

FIG. 1. Purification scheme. Summary of steps of the purification showing average percentage distribution of activity from the preceding fraction.

Recovered by recombination of the pellet and the supernatant solution.

The addition of digitonin for enzyme solubilization caused a 1.5- to 2-fold activation. The enzyme became unstable in the presence of digitonin and lost activity as shown in Fig. 2. The half-life of the enzyme after the addition of digitonin is approximately 30 hours. However, the digitonin supernatant solution lost no more than 25% of the activity after 1 month at -70°. Triton X-100 solubilized the enzyme to approximately the same extent as digitonin.

The instability of nitrate reductase in digitonin was observed in all succeeding fractions until ammonium sulfate was mixed with Fraction VI. In the presence of ammonium sulfate, the enzyme could be stored at 0° for several months without signifi-
TABLE I
Summary of purification of nitrate reductase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg/ml)</th>
<th>Activity* (units/ml)</th>
<th>Specific activity (units/mg protein)</th>
<th>Total activity (units)</th>
<th>Recovery of units %</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. 3,000 X g supernatant</td>
<td>7.2</td>
<td>32,800</td>
<td>4,550</td>
<td>1,640,000</td>
<td>61</td>
</tr>
<tr>
<td>II. 144,000 X g pellet</td>
<td>15.6</td>
<td>99,600</td>
<td>6,380</td>
<td>996,600</td>
<td>76</td>
</tr>
<tr>
<td>(144,000 X g supernatant)</td>
<td>4.0</td>
<td>4,570</td>
<td>91,000</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>III. Digitonin suspension</td>
<td>7.8</td>
<td>74,640</td>
<td>9,570</td>
<td>1,492,920</td>
<td>91</td>
</tr>
<tr>
<td>IV. Digitonin supernatant</td>
<td>2.9</td>
<td>62,000</td>
<td>21,275</td>
<td>1,240,000</td>
<td>76</td>
</tr>
<tr>
<td>V. Calcium phosphate supernatant</td>
<td>2.3</td>
<td>57,600</td>
<td>25,530</td>
<td>1,153,200</td>
<td>70</td>
</tr>
<tr>
<td>VI. Calcium phosphate eluate</td>
<td>1.2</td>
<td>48,870</td>
<td>41,400</td>
<td>350,880</td>
<td>30</td>
</tr>
<tr>
<td>VII. Ammonium sulfate supernatant</td>
<td>0.4</td>
<td>9,370</td>
<td>25,530</td>
<td>137,990</td>
<td>8</td>
</tr>
<tr>
<td>VIII. Ammonium sulfate pellet</td>
<td>2.5</td>
<td>103,200</td>
<td>41,400</td>
<td>350,880</td>
<td>21</td>
</tr>
</tbody>
</table>

* One unit of activity is defined as 1 Klett unit, which, according to a standard curve, represents 9.52 \times 10^{-7} \text{mole of nitrite.}

The numbers are the difference in Klett units between the 5-minute enzymatic reaction and the zero time control.

Fig. 2. Instability of the digitonin supernatant. The conditions of the standard assay were used. The digitonin-treated enzyme preparation (Fraction IV) was stored at a concentration of 4 mg of protein per ml of 0.1 M acetate buffer, pH 6.0, and appropriately diluted for analysis of activity as a function of time.

TABLE II
Loss of activity in presence and absence of ammonium sulfate

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Acetate buffer</th>
<th>Acetate plus (NH₄)₂SO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity</td>
<td>Lost</td>
<td>Specific activity</td>
</tr>
<tr>
<td>units/mg protein</td>
<td>%</td>
<td>units/mg protein</td>
</tr>
<tr>
<td>0</td>
<td>27,950</td>
<td>23,750</td>
</tr>
<tr>
<td>23</td>
<td>26,200</td>
<td>25,100</td>
</tr>
<tr>
<td>37</td>
<td>14,870</td>
<td>24,900</td>
</tr>
</tbody>
</table>

Sedimentation Studies in Relation to Stability—Examination of Fraction VIII in the Beckman model E analytical ultracentrifuge suggests that instability is correlated with the disintegration of a large particle. The enzyme was removed from ammonium sulfate by centrifugation and resuspended in 0.1 M acetate buffer, pH 6.0. To half the resuspended enzyme, 40% ammonium sulfate was added. Samples of each fraction were examined at various times in the analytical ultracentrifuge and concurrently assayed for activity. The results of this experiment are summarized in Table II and Fig. 3.

Table II shows that the specific activity of the nitrate reductase, without added ammonium sulfate, drops to 79% of its original value over the 38-hour duration of the experiment whereas the specific activity in the presence of ammonium sulfate remains constant.

cant loss of activity. Removal of the precipitated enzyme by centrifugation (Fraction VIII) resulted in the same instability exhibited in the digitonin supernatant solution (Fraction VI). Readdition of ammonium sulfate during the inactivation stabilized but did not restore activity.

There are some indications that enzyme stability is directly related to enzyme dilution. At low concentrations (in the range of 100 \mu g of protein per ml), activity is stable with and without added ammonium sulfate. At higher concentrations, however, instability increased and the activity was stable only in the presence of ammonium sulfate. It was not determined whether the instability was due to an increase in protein or digitonin concentration.

Occasionally an activation of the nitrate reductase, at times as large as 10-fold, was observed at the ammonium sulfate stage of the preparation. In these unusual preparations, the specific activity of Fractions I to VI was always smaller (by an order of 10-fold) than the more usual preparations. Total activation of these preparations by ammonium sulfate required about 1 week, and the extent of the activation was such that the final specific activity of these unusual preparations was identical with that of the more usual preparations.

As a result of the various activations (digitonin and occasionally ammonium sulfate) and losses of activity (irreversible loss on centrifugation at 144,000 X g, instability after digitonin addition), the actual purification cannot be calculated but is probably higher than the observed increase in specific activity of Fraction VIII over the crude sonic extract.
Fig. 3 shows the corresponding protein peaks seen in the ultracentrifuge with and without added ammonium sulfate. At zero time and at 2½ hours after resuspension (Fig. 3A), two major peaks are visible with sedimentation coefficients (corrected to water at 20°) of 16 to 17 S and 7 to 8 S. Separation of the peaks by means of a moving partition cell allowed a correlation of activity with the faster of these two components. In addition to these two peaks, a third, minor component is observed without ammonium sulfate, located between the fast and slow peaks with a sedimentation coefficient of 13 S.

Fig. 3B shows the sedimentation patterns obtained 13½ hours after resuspension of the pellet. Without added ammonium sulfate, the 16 to 17 S active peak decreased in size with a concomitant increase in the 13 S peak. This phenomenon was not observed in the presence of ammonium sulfate; the relative sizes of the two peaks as well as the activity remained constant. At 37½ hours (Fig. 3C), the peaks shifted still further in acetate buffer but remained unchanged in the presence of ammonium sulfate. Urea completely inhibits enzymatic activity within 30 minutes of preincubation. As shown in Fig. 3D, only one peak is seen in 4 M urea with a sedimentation coefficient corrected to water at 20° of 9 to 10 S.

These data suggest that activity is correlated with particle size and that instability is probably related to the disintegration of a large particle into smaller components. The stabilizing effect of ammonium sulfate on particle size is directly correlated with its effect on activity.

Cytochrome Components—Difference spectra (enzyme reduced with dithionite minus untreated enzyme) of the various fractions obtained during purification have revealed the presence of several cytochrome components. Peaks at 550, 520, and 418 m show the a-, α,-, and α types of cytochrome c, respectively. Those seen at 589, 605, and 437 m indicate a composite of cytochrome a peaks. The 589 m peak probably represents a cytochrome a-type component, and the 605 m peak a cytochrome a-type component. The small shoulder at 530 mF may be indicative of cytochrome b.

Fig. 4A shows the difference spectrum of Fraction I and indicates the presence of cytochromes a, a, c, and possibly b. The height of the a-peak for cytochrome c at 550 mF is greater than that of the cytochrome a types represented by absorption from 585 to 610 mF.

Much of the cytochrome c in Fraction I is soluble in nature and remains in the 144,000 x g supernatant solution. The spectra of Factions II, III, IV, V, and VI are essentially identical and are typified by the difference spectrum of Fraction IV (Fig. 4B). The height of the peaks for cytochrome c at 550 mF and for cytochrome a at 589 mF are now approximately equal, and the prominent shoulder at 605 mF shows the presence of cytochrome a.

Fig. 5 compares the difference spectra of Factions VII and VIII. Ammonium sulfate treatment apparently cleaves the 605 mF cytochrome from the pellet. The a-absorption peak of the cytochrome a types differs in the two fractions, that of the pellet being located at about 436 mF (cytochrome a) and that of the supernatant solutions at 439 mF (cytochrome a). Spectra obtained from Fraction VIII at liquid nitrogen temperature show similar features. At these low temperatures, however, the cytochrome a and a peaks are present in Fraction VIII.

The presence of cytochrome b in these preparations is suggested by the shoulder at 530 mF and has been confirmed by preparing the pyridine-hemochromogen derivative of ether extracts of lyophilized samples of Factions I and VIII according to the method of Jacobs and Wolin (17). The extracts showed absorption peaks at 555 and 524 mF, which are attributed to a cytochrome b component (17, 18).
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Proportionality and pH Optimum—The rate of enzymatic nitrate reduction is linear with time and proportional to protein concentration provided that the cytochrome concentration is appropriately varied (see below). The activity of both nitrate reductase and cytochrome oxidase with reduced cytochrome c as donor is optimal between pH 5.5 and 6.5, and falls sharply above and below these values (Fig. 6).

Substrate Affinity—The apparent $K_m$ for nitrate, as estimated from the saturation curve, is $4 \times 10^{-4}$ M (Fig. 7A). Some substrate inhibition is observed above $10^{-3}$ M nitrate (not shown). The affinity of the enzyme for reduced cytochrome c was identical whether determined by nitrite appearance or the rate of oxidation of reduced cytochrome c. Fig. 7B shows that substrate inhibition occurs at cytochrome concentrations above that required for optimal activity. Moreover, the optimal cytochrome concentration decreases with decreasing enzyme concentration. The apparent $K_m$, although variable with protein concentration, is in the range of $10^{-4}$ M reduced cytochrome c.

Electron Donors of System—Other electron donors for the enzymatic reduction of nitrate to nitrite include the reduced forms of 2,6-dichlorophenolindophenol, 2,3',6-trichlorophenolindophenol, benzyl and methyl viologen, methylene blue, and toluidine blue. Optimal conditions with leucoindophenol are essentially the same as those with reduced cytochrome c as donor except that the pH optimum is between 6.0 and 7.0 and the estimated $K_m$, which does not vary with protein concentration, is $1.5 \times 10^{-4}$ M leucoindophenol.

Effect of Inhibitors—The effect of various inhibitors on nitrate reductase activity is shown in Table III. Electron transport inhibitors such as urethane, sodium Amytal, and atebrine had no effect on enzymatic activity whereas antimycin A and 2-n-heptyl-4-hydroxyquinoline N-oxide caused some degree of inhibition which increased with preincubation.

Both cyanide and azide were equally inhibitory at low concentrations. Inhibition by azide was unaffected by the order of addition of reagents, whereas the degree of inhibition by cyanide was markedly influenced by whether or not nitrate was added to the enzyme prior to the addition of cyanide. For example, at $5 \times 10^{-4}$ M cyanide, preincubation of enzyme with cyanide alone resulted in 100% inhibition, whereas preincubation with both cyanide and nitrate produced only 17% inhibition (Fig. 8A). This protective effect of nitrate against cyanide inhibition occurred only if nitrate was present before the enzyme was mixed with cyanide. Under these conditions, cyanide is apparently competitive with nitrate at concentrations as high as $10^{-3}$ M cyanide (Fig. 8B). The slight differences in $V_{max}$ are replicable and may be ascribed either to instability of the enzyme during the experiment or to a noncompetitive effect as well in the inhibition by cyanide.

Cyanide at a concentration of $5 \times 10^{-4}$ M combines with one or both of the cytochrome $a_1$ components, since the cyanide
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Metal-binding agents other than cyanide, azide, and carbon monoxide which affected activity were o-phenanthroline, \( \alpha, \alpha' \)-dipyridyl, salicylaldoxime, and 8-hydroxyquinoline, the percentage inhibition increasing with preincubation (Table III). EDTA was not as effective an inhibitor. Diethyldithiocarbamate was a potent inhibitor, although the degree of inhibition may be subject to error because the metal chelator not only had a significant effect on color development but also caused the formation of nitrite on preincubation with enzyme and nitrate.

The above inhibition by metal-binding agents suggests involvement of a metal in nitrato reduction. That a metal is involved in nitrato reductase and probably not in cytochrome oxidase activity is indicated in Table IV. Cytochrome oxidase is slightly or not at all affected by \( \alpha, \alpha' \)-dipyridyl and salicylaldoxime, whereas nitrato reductase is inhibited to a large extent. Separate experiments with the oxygen electrode also show that neither of these reagents prevents oxygen uptake. Cyanide and azide, however, strongly affect both activities, suggesting another site of action of metal-binding agents.

A lyophilized sample of the final enzyme preparation (Fraction VIII) was subjected to preliminary analysis by electron spin resonance studies. The resulting signals indicated a low ferrie iron content and the presence of several unknown free radical signals at 3000 gauss. The preparation does not contain manganese or copper in the cupric state.

No metals have been found to stimulate activity, although several inhibit at high concentrations. After a 30-minute preincubation with \( 5 \times 10^{-2} \) M barium, calcium, cobalt, nickel, cadmium, zinc, manganese, or chromate salts, 30 to 70% inhibition of nitrato reductase activity occurred. Sodium fluoride and 4 M urea were likewise inhibitors of nitrato reductase.

Iodoacetate and N-ethylmaleimide did not inhibit nitrato reductase, but p-hydroxymercuribenzoate was found to inhibit at high concentrations with preincubation slightly increasing the inhibition.

Glutathione (reduced), mercaptoethanol, cysteine, and cystine had no apparent effect on nitrato reductase. However, these reagents caused the nonenzymatic disappearance of nitrite, which could be partially avoided by oxidizing the resulting hydroxylamine to nitrite with iodine and then destroying excess iodine with arsenite (19). Apparently, then, sulfhydryl reagents affect the nonenzymatic reduction of nitrite to hydroxylamine. That not all of the nitrite was recovered by this method may reflect an additional effect of these reagents on color development. Also, nitrite was formed when the sulfhydryl reagent, nitrate, and enzyme were combined, suggesting that sulfhydryl function as electron donors for the system.

Chlorate ion inhibits nitrato reductase and also oxidizes cytochrome c enzymatically (Table III). The inhibition by chlorate appears to be of a competitive nature (Fig. 9) although the \( V_{\text{max}} \) is not exactly identical for each chlorate concentration. This may be due to slight losses of activity during the course of the experiment or to the generation of chloride as chlorate is reduced. Chlorite ion is known to destroy cytochromes (20), which would introduce a noncompetitive element into the observed inhibition.

**Stoichiometry.—**The expected ratio of moles of cytochrome c oxidized to moles of nitrite formed is 2. Ratios greater than this value reflect cytochrome oxidase activity, also present in Fraction VIII, in addition to nitrato reductase activity. Table V shows that without added nitrate the ratio is high, indicating that most of the electrons serve to reduce oxygen and not nitrate.
The predominant activity is therefore cytochrome oxidase. Some nitrate reductase activity also occurs since nitrite is formed, apparently arising from the nondialyzable, endogenous nitrate present in Fraction VIII. Addition of nitrate to the reaction mixture reduced the ratio from greater than 30 to values of 2 or 3, showing that the predominant activity was now nitrate reductase, even in the presence of oxygen. Separate experiments with the oxygen electrode have also shown that nitrate in the reaction mixture actually prevents oxygen uptake.

Carbon monoxide does not inhibit nitrate reductase activity, and the stoichiometry remains essentially unchanged with added nitrate (Table VI). Without added nitrate, when the predominant activity is that of cytochrome oxidase (as indicated by the aerobic ratio of 35.5), carbon monoxide decreases the amount of cytochrome oxidized by inhibiting cytochrome oxidase (but not nitrate reductase) and the stoichiometry consequently drops to a value of 2.8.

Fig. 10 shows the stoichiometry of nitrate reduction as a function of nitrate concentration. The ratio without added nitrate is 25 but drops steeply as nitrate is added, reaching a value of 3.9 at the $K_m$ for nitrate (estimated at $2.5 \times 10^{-3}$ M in this preparation) and a final ratio of 3.0 at concentrations above $10^{-4}$ M nitrate.

Nitrite formation and stoichiometry have also been examined as a function of reduced cytochrome c concentration for three different dilutions of the enzyme preparation (Fig. 11). The curves obtained for nitrite appearance (Fig. 11A) clearly illustrate (a) substrate inhibition above optimal cytochrome c concentrations and (b) a decrease of optimal cytochrome concentration with decreasing protein. The corresponding curves for stoichiometry (Fig. 11B) show a steady increase in the ratio of cytochrome c oxidized to nitrite formed as the concentration of reduced cytochrome c is increased. At each protein concentration, the ratio is 2 at optimal concentrations of reduced cytochrome c and ranges from 2 to 3 throughout the region of maximum nitrate reductase activity. Above optimal cytochrome concentrations, the ratio becomes progressively larger, increasing with the concentration of cytochrome c. The experiment indicates that in the presence of nitrate and optimal cytochrome concentrations, only the nitrate reductase is functioning; at excess cytochrome concentrations, however, additional reactions are occurring.

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Investigations to determine the nature of these additional reactions at elevated cytochrome concentrations have suggested at least two possibilities. First, there is a nonenzymatic disappearance of nitrite dependent upon the concentration of both nitrite and reduced cytochrome c. The disappearance of nitrite was insignificant at optimal cytochrome concentrations but became significant as the cytochrome level was increased. No nitrite reductase activity was present. Second, although under conditions of optimal cytochrome concentration nitrate was found to inhibit oxygen uptake completely, increasing concentrations of reduced cytochrome c resulted in a progressively increased oxygen uptake, although never to the extent seen without added nitrate. Deviations from the ratio of 2, then, could also be partly due to cytochrome oxidase activity.

Cytochrome Involvement—The involvement of a cytochrome pathway in enzymatic nitrate reduction in *Nitrobacter agilis* is suggested by the presence of cytochromes in the preparation and by the electron donor role of reduced cytochrome c in the enzymatic reduction of nitrate. Direct evidence for cytochrome participation would consist of reduction of bacterial cytochromes by reduced cytochrome c and their reoxidation by nitrate concomitant with nitrite appearance. In view of the interfering absorption of reduced cytochrome c at 550 m$_u$, dithionite was selected as electron donor.

Addition of nitrate to dithionite-reduced Fraction VIII resulted in the reoxidation of its cytochrome c and part of the cyto-
Correlation of these spectra with nitrite appearance is presented in Table VII. Neither carbon monoxide nor molecular nitrogen had an effect on nitrite appearance, although carbon monoxide completely inhibited cytochrome oxidase activity under these conditions. As a control, the enzyme was inactivated (as determined with reduced cytochrome c as electron donor) for 2 minutes at 70°C and similarly treated with dithionite and nitrate. The reduced cytochrome peaks of the heat-inactivated enzyme disappeared at a considerably slower rate, and the corresponding rate of nitrite production decreased 90%.

Dithionite-reduced cytochromes of Fraction VII show a predominance of the 605 μM cytochrome a absorption peak. Addition of excess nitrate resulted in the oxidation of cytochrome c and most of the cytochrome a at 589 μM. However, cytochrome a at 605 μM remained reduced, suggesting that it is not involved in nitrate reduction.

The above studies with Fraction VIII were repeated with spectral examination at liquid nitrogen temperatures, where the α-
peak of cytochrome a1 is observed as two components with absorption peaks at 587 and 583 μm. Fig. 12 shows that addition of nitrate results in the complete disappearance of the reduced absorption peaks of bacterial cytochrome c and one of the two cytochrome a1 components. The other cytochrome a1 component, located at 583 μm, is apparently not involved in nitrate reductase activity. Oxygen added in place of nitrate likewise did not reoxidize the 583 μm absorption peak, showing that it is similarly not involved in cytochrome oxidase activity. In the presence of carbon monoxide, nitrate, but not oxygen, reoxidized these same absorption peaks of the dithionite-reduced preparation (Fig. 12). The effects of carbon monoxide on the Soret region are illustrated in Fig. 13. Although carbon monoxide combines with bacterial cytochrome, as evidenced by the carbon monoxide difference spectrum, it does not prevent the disappearance of the γ-peak of the cytochrome a-type components upon addition of nitrate.

**DISCUSSION**

Two major types of nitrate reductase enzymes are distinguished in the literature: (a) the assimilatory type, which does not involve the cytochromes and which reduces nitrate for the biosynthesis of nitrogen-containing components (for example, amino acids and nucleic acids), and (b) the respiratory or nonassimilatory type, whereby nitrate serves as a terminal electron acceptor similar to aerobic respiration (8, 9). Respiratory nitrate reduction requires participation of a cytochrome, although the cytochromes involved vary according to the organism, and the products of such nitrate reduction are generally excreted from the cell.

The assimilatory type of nitrate reductase is found in aerobes such as *Neurospora* and in green plants. Nason and Evans (21) and Nicholas and Nason (22) have shown that the nitrate reductase of *Neurospora crassa* involves the transfer of electrons from TPNH to flavin to molybdate to nitrate. The enzyme obtained from soybeans is essentially identical except that both TPNH and DPNH function as electron donors (23, 24).

Of the respiratory type of nitrate reductases, the most extensively studied are those of *Escherichia coli* and *Achromobacter fischeri.* A number of workers (25–28) have demonstrated that nitrate reduction in *E. coli* involves the participation of cytochrome b1. The enzyme has been purified to a homogeneous form, has a molecular weight of 10^6, contains 1 atom of molybdenum and 40 atoms of iron, and will reoxidize cytochrome b1 anaerobically in the presence of nitrate, a process inhibited by quinoline oxide. In *A. fischeri,* the pathway of electron transport has been shown to be from TPNH (or DPNH) to FAD (or flavin mononucleotide) to iron to cytochrome c to either oxygen or nitrate (29). The enzyme has recently been purified and the cytochrome c removed, so that the enzyme preparation now shows only a protein peak at 280 μm. Neither flavins nor various metals (including iron and molybdenum) have been found to stimulate activity, and only a reduced dye will serve as donor for enzymatic nitrate reduction (30).

*Nitrobacter agilis* contains a respiratory type of nitrate reduc-

---

**TABLE III**

**Effect of inhibitors on nitrate reductase**

The conditions of the standard assay were used. About 4 to 9 μg of protein were added to each inhibitor, and the reaction was started either immediately or after 30 minutes by the addition of nitrate and reduced cytochrome c. The observed inhibition was started either immediately or after 30 minutes by the addition of nitrate.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Final concentration</th>
<th>Inhibition</th>
<th>30-minute preincubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Urethane</td>
<td>5 × 10^-3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sodium Amytal</td>
<td>5 × 10^-3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2-n-Heptyl-4-hydroxy-</td>
<td>5 × 10^-4</td>
<td>8</td>
<td>37</td>
</tr>
<tr>
<td>quinoline N-oxide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antimycin A</td>
<td>2 × 10^-2</td>
<td>50</td>
<td>89</td>
</tr>
<tr>
<td>Atebrine</td>
<td>2 × 10^-2</td>
<td>22</td>
<td>59</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>5 × 10^-5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Potassium cyanide*</td>
<td>5 × 10^-5</td>
<td>8</td>
<td>93 (-NO3^-)</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>5 × 10^-4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Carbon monoxide</td>
<td>5 min bubbling</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>α-Phenanthroline</td>
<td>5 × 10^-3</td>
<td>96</td>
<td>89</td>
</tr>
<tr>
<td>α,α′-Dipyridyl</td>
<td>5 × 10^-3</td>
<td>21</td>
<td>72</td>
</tr>
<tr>
<td>Salicyaldoxime</td>
<td>5 × 10^-3</td>
<td>51</td>
<td>100</td>
</tr>
<tr>
<td>S-Hydroxyquinoline</td>
<td>5 × 10^-2</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>5 × 10^-3</td>
<td>20</td>
<td>66</td>
</tr>
<tr>
<td>Diethyldithiocarbamate</td>
<td>5 × 10^-4</td>
<td>32</td>
<td>37</td>
</tr>
<tr>
<td>Idoic acid</td>
<td>5 × 10^-2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>5 × 10^-2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>p-Methoxymercuribenzoate</td>
<td>5 × 10^-3</td>
<td>53</td>
<td>62</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>5 × 10^-3</td>
<td>56</td>
<td>76</td>
</tr>
<tr>
<td>Sodium fluoride</td>
<td>5 × 10^-3</td>
<td>0</td>
<td>41</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4</td>
<td>55</td>
<td>97</td>
</tr>
</tbody>
</table>

* Without preincubation, enzyme was added to a reaction mixture containing both nitrate and cyanide. For a 30-minute preincubation, enzyme was added to a reaction mixture containing cyanide in either the absence or presence of added nitrate.

---

**Scheme 1**

```
 **KCN**

 KCN

 KCN

metal (?) → nitrate

 cytochrome c (583 μm) → (?)

 cytochrome c → cytochrome a (587 μm)

 cytochrome a (605 μm) → O2

 CO

Scheme 1
```
Nitrate Reductase from Nitrobacter agilis

Fig. 8. A, effect of cyanide and azide concentration on nitrate reductase activity. The conditions of the standard assay were used. For "no preincubation," enzyme (5 µg of Fraction VIII) was added to a reaction mixture containing nitrate and inhibitor, and the reaction was started immediately by the addition of reduced cytochrome c. With a 30-minute preincubation, enzyme was first preincubated with inhibitor in the presence and absence of nitrate, and the reaction was finally started by the addition of reduced cytochrome c (and nitrate in the case of preincubation without nitrate) to the preincubation mixture. Under each of these three conditions, the curves obtained with azide were identical. The results are presented on a semilogarithmic plot. B, Lineweaver-Burk plot showing competitive inhibition between cyanide and nitrate. The conditions of the standard assay were used. Each line was obtained by the addition of 4 µg of enzyme (Fraction VIII) to a reaction mixture containing reduced cytochrome c and the designated concentrations of cyanide and nitrate.

Table IV

Effect of metal chelators on nitrate reductase and cytochrome oxidase

Approximately 10 µg of protein (Fraction VIII) were preincubated in the presence and absence of each inhibitor for 30 minutes before the addition of reduced cytochrome c. The oxidation of cytochrome was measured by the decrease in optical density at 550 nm. At the end of 5 minutes, the reaction was stopped by the addition of sulfanilic acid and each cuvette was examined for appearance of nitrite. The predominant activity is that of cytochrome oxidase, since no nitrate was added to the reaction mixture. The nitrate reductase activity present, as determined by nitrite appearance, is due to endogenous, nondialyzable nitrate. Similar experiments with added nitrate showed the same inhibition of nitrite appearance.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Nitrite appearance</th>
<th>Cytochrome c oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount</td>
<td>Inhibition</td>
</tr>
<tr>
<td></td>
<td>µmoles × 10^−6</td>
<td>%</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salicylaldoxime, 10^−3 M</td>
<td>0.38</td>
<td>39</td>
</tr>
<tr>
<td>None</td>
<td>1.42</td>
<td>67</td>
</tr>
<tr>
<td>α,α'-Dipyridyl, 5 × 10^−5 M</td>
<td>0.47</td>
<td>67</td>
</tr>
</tbody>
</table>

tase of particulate nature. The proposed pathway of electron transport for nitrate reduction in Nitrobacter agilis has been derived from spectral and inhibition studies reported in the present paper and is illustrated in Scheme 1. Electrons are transferred from bacterial cytochrome c to both cytochrome α components, namely, those at 583 and 587 nm (Figs. 5 and 12). The function...
of the cytochrome $a_1$ at 583 mp is unknown, whereas the cytochrome $a_1$ at 587 mp is involved in electron transport. Since both nitrate and oxygen oxidize bacterial cytochromes $c$ and $a_1$ at 587 mp (Fig. 12), these two components are placed in a position common to both nitrate reductase and cytochrome oxidase activity. From cytochrome $a_1$ (587 mp), electrons may be donated either directly to nitrate or through cytochrome $a$ (605 mp) to oxygen.

Several lines of evidence indicate that cytochrome $a$ is involved only in oxidase activity. After ammonium sulfate precipitation, 80% of the nitrate reductase activity is found in the resuspended pellet but most of the cytochrome $a$ remains in the supernatant solution (Fig. 5). At this stage of the purification the ratio of nitrate reductase to cytochrome oxidase activity increases several fold in the pellet. Moreover, in Fraction VII, the 605 mp cytochrome $a$ absorption peak is not reoxidized by nitrate. Finally, carbon monoxide combines with a cytochrome $a$-type component (Fig. 13) and inhibits cytochrome oxidase activity completely but has no effect on nitrate reductase activity (Figs. 12 and 13; Tables III, VI, and VII).

### Table V

Aerobic stoichiometry of nitrate reductase with and without added nitrate

Cytochrome oxidation was measured by the decrease in optical density at 550 mp. At the designated time, sulfanilic acid was added to the cuvettes for nitrite determination. Enzymatic nitrite formation in the absence of added nitrate is due to endogenous nondialyzable nitrate present in Fraction VIII. The ratio of micromoles of cytochrome oxidized to micromoles of nitrite formed (B:A) represents the resulting stoichiometry in the presence and absence of added nitrate. Each reaction mixture contained 6 μg of protein.

<table>
<thead>
<tr>
<th>Added nitrate</th>
<th>Length of reaction</th>
<th>Nitrite formed (μmoles)</th>
<th>Cytochrome oxidized (μmoles)</th>
<th>B:A</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>2.5</td>
<td>0.28</td>
<td>10.2</td>
<td>36.5</td>
</tr>
<tr>
<td>+</td>
<td>2.5</td>
<td>5.63</td>
<td>23.9</td>
<td>2.7</td>
</tr>
<tr>
<td>+</td>
<td>3.5</td>
<td>0.62</td>
<td>23.9</td>
<td>3.7</td>
</tr>
</tbody>
</table>

### Table VI

Stoichiometry of nitrate reductase under atmospheres of air and carbon monoxide

The conditions of the standard assay were used with 4 μg of protein. Cytochrome oxidation was determined by the decrease in optical density at 550 mp. The reaction was terminated after 3 minutes by the addition of sulfanilic acid, and the contents of each cuvette were analyzed for appearance of nitrite. For carbon monoxide data, the reaction mixtures, contained in Thunberg cuvettes, were evacuated, flushed several times with nitrogen gas, and filled with carbon monoxide.

<table>
<thead>
<tr>
<th>Atmosphere</th>
<th>Stoichiometry*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without nitrate</td>
<td>35.5</td>
</tr>
<tr>
<td>With nitrate</td>
<td>3.7</td>
</tr>
<tr>
<td>Carbon monoxide</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>3.7</td>
</tr>
</tbody>
</table>

* Ratio of micromoles of cytochrome c oxidized to micromoles of nitrite formed.

**Fig. 10.** Stoichiometry as a function of nitrate concentration. Curves were obtained with 8 μg of protein and 2.2 X 10⁻³ m reduced cytochrome c. The amount of cytochrome oxidized as a function of nitrate concentration (∆---∆) was determined by the decrease in optical density at 550 mp. The reaction was stopped after 5 minutes by the addition of sulfanilic acid, and the contents of each cuvette were analyzed for nitrite appearance (∆---∆). The ratio of micromoles of cytochrome c oxidized to micromoles of nitrite formed is shown for each nitrate concentration employed (∆---∆).
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**FIG. 11.** Stoichiometry as a function of reduced cytochrome c concentration. Curves were obtained with 10^{-4} m final concentration of nitrate for 2, 3, and 6 μg of protein as indicated. The amount of cytochrome oxidized at each concentration of reduced cytochrome c was determined by the decrease in optical density at 550 mp. The reaction was stopped after 3½ minutes by the addition of sulfanilic acid, and the contents of each cuvette were analyzed for nitrite appearance (A). The ratio of micromoles of cytochrome oxidized to micromoles of nitrite formed was determined for each concentration of reduced cytochrome c (B).

**TABLE VII**

**Nitrate reductase activity with dithionite as electron donor**

The conditions of the standard assay were used with approximately 1.0 mg of protein per reaction mixture and dithionite (0.1 ml of a 0.1% solution in 0.1 m phosphate buffer, pH 7.0) as electron donor. Aliquots were removed from each cuvette at 2-minute intervals and assayed for nitrite appearance. Each 1-ml reaction mixture was concurrently examined spectrally in the Cary model 14 spectrophotometer.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NO_{3}^{-} formed</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μ mole/min</td>
<td>%</td>
</tr>
<tr>
<td>None</td>
<td>2.60 	imes 10^{-4}</td>
<td>0</td>
</tr>
<tr>
<td>2 minutes at 70\degree</td>
<td>0.27 	imes 10^{-4}</td>
<td>90</td>
</tr>
<tr>
<td>CO for 3 minutes</td>
<td>2.54 	imes 10^{-4}</td>
<td>0</td>
</tr>
<tr>
<td>N__ for 3 minutes</td>
<td>2.48 	imes 10^{-4}</td>
<td>0</td>
</tr>
</tbody>
</table>

Inhibition studies with metal chelators have implicated an as yet unidentified metal component in the nitrate reductase system which is not involved in cytochrome oxidase activity (Table IV). The present investigations with cyanide (Fig. 7) and established metal-binding agents (Table III) suggest that both compounds are competing for a common site which may be the unknown metal. That cyanide also combines with a cytochrome a component has been demonstrated spectrally, and, accordingly, cyanide inhibits both cytochrome oxidase and nitrate reductase activity.

The enzyme is probably a component of a high molecular weight particle as evidenced by the sedimentation coefficient of 17 S for the active peak. Such a high sedimentation coefficient probably places the molecular weight of the active peak in the millions. Since it is currently accepted that bacterial systems contain electron transport particles, the action of digitonin may be in the solubilization of these particles. Apparently the disruption of the integral structure of the particle causes its inactivation; the active 17 S peak is converted to a 13 S peak concurrent with loss of nitrate reductase activity.

Of particular interest is the fact that oxygen is not inhibitory to nitrate reduction in this preparation from *Nitrobacter agilis*. This is in contrast to all other respiratory-type nitrate reductases.

**FIG. 12.** Effects of nitrate (in the presence and absence of carbon monoxide) and oxygen on dithionite-reduced Fraction VIII. Spectra were obtained with 1.0 mg of protein per ml of 0.1 m acetate buffer, pH 6.0, in the split beam spectrophotometer at liquid nitrogen temperatures. A solution of enzyme was titrated with dithionite (in 0.1 m phosphate buffer, pH 7.0) until just reduced as determined on a dual wave length spectro-photometer. An aliquot was removed, and the difference spectrum (enzyme with dithionite minus untreated enzyme) was recorded at liquid nitrogen temperatures (---). To the remainder of the reduced enzyme solution, nitrate (---) or oxygen (-----) was added, and the spectrum was recorded after completion of the reaction again as determined with the dual wave length spectro-photometer. An aliquot was removed, and the difference spectrum (enzyme with dithionite minus untreated enzyme) was recorded at liquid nitrogen temperatures (---). To the remainder of the reduced enzyme solution, nitrate (---) or oxygen (-----) was added, and the spectrum was recorded after completion of the reaction again as determined with the dual wave length spectrophotometer. The experiment was repeated in the presence of carbon monoxide, and the spectrum was recorded after addition of nitrate to the carbon monoxide-treated, dithionite-reduced enzyme (---).
In *E. coli*, for example, no nitrate reductase activity is detected unless the reaction is determined anaerobically (25). In the case of the *Nitrobacter* enzyme, not only does oxygen fail to decrease nitrate reductase activity, but the addition of nitrate to the reaction mixture actually prevents oxygen uptake.

Since the metabolism of *Nitrobacter agilis* depends on the oxidation of nitrite to nitrate, it is of interest that this organism also possesses an enzyme capable of reducing nitrate to nitrite as reported here. Although the physiological significance of this enzyme is not entirely clear, the reduction could be the first step of a sequence for providing nutritional nitrogen, or it could be a means of recycling the nitrite used for nitrite oxidation. The latter possibility would result in increasing the low efficiency (5 to 10%) of nitrite oxidation in *Nitrobacter*. The nitrate reduction of nitrite to nitrate, it is of interest that this organism also possesses an enzyme capable of reducing nitrate to nitrite as reported here. Although the physiological significance of this enzyme is not entirely clear, the reduction could be the first step of a sequence for providing nutritional nitrogen, or it could be a means of recycling the nitrite used for nitrite oxidation.

The analyses presented in this paper shed some light on this problem by the demonstration that chlorate competitively inhibits the enzymatic reduction of nitrate to nitrite.

**SUMMARY**

A heat-labile, particulate enzyme which catalyzes the reduction of nitrate to nitrite with reduced cytochrome c as electron donor has been solubilized and partially purified from the chemotroph *Nitrobacter agilis*.

Solubilization by treatment with digitonin resulted in a marked instability of the enzyme. The analytical ultracentrifuge revealed two major protein peaks with sedimentation coefficients of 17 S and 7 S, and a minor component with a sedimentation coefficient of 15 S. Activity was located in the 17 S peak. Instability was correlated with a shift in concentration of two protein peaks, the 17 S component decreasing with time and the 13 S peak concurrently increasing. Ammonium sulfate was found to stabilize both the activity and the disintegration of the 17 S component.

Cytochrome oxidase activity was also present in the preparation. Nitrate, however, was found to interfere with cytochrome oxidase activity since aerobically the ratio of moles of cytochrome a oxidized per mole of nitrite formed ranged from 2 to 3. Separate experiments with the oxygen electrode have also established that nitrate does, in fact, prevent oxygen uptake.

The enzyme preparation contains cytochromes c, b, a, and a1; the absorption peak at 559 mμ, indicative of cytochrome a1, is represented by two components at 587 and 583 mμ when examined spectrally at liquid nitrogen temperatures. Spectral studies have shown that the pathway for nitrate reduction involves cytochromes c and one of the a1 components, namely that with an absorption peak at 587 mμ, whereas the pathway for cytochrome oxidase activity involves these same cytochromes and, in addition, cytochrome a at 605 mμ. The function of the other cytochrome a1 component (583 mμ) is unknown.

Cyanide and azide inhibited both nitrate reductase and cytochrome oxidase activity. The protective effect of nitrate against inhibition by cyanide is competitive. Cyanide also combines with a cytochrome a1 component as determined by spectral analysis. Carbon monoxide did not inhibit nitrate reductase activity although it did combine with a cytochrome a-type component and completely inhibited cytochrome oxidase activity.

Finally, the competitive inhibition between nitrate and chlorate merits comment. In 1945, Lees and Quastel (31) noted that the addition of chlorate to growing cultures of *Nitrobacter* delayed growth but did not affect nitrite oxidation in established populations. Nitrate was found to abolish the chlorate effect. In addition, chlorate at higher concentrations inhibited nitrite oxidation, but this effect was not abolished by added nitrate. Lees suggested that "*Nitrobacter* uses nitrate rather than nitrite as a source of nutritional nitrogen and that chlorate somehow blocks the utilization of nitrate" (20). The results with chlorate presented in this paper shed some light on this problem by the demonstration that chlorate competitively inhibits the enzymatic reduction of nitrate to nitrite.

**Fig. 13.** Effects of nitrate in the presence of carbon monoxide on dithionite-reduced Fraction VIII. Spectra were obtained with 1.0 mg of protein per ml of 0.1 m acetate buffer, pH 6.0, in the split beam spectrophotometer at temperatures of liquid nitrogen. A solution of enzyme was titrated with dithionite (in 0.1 m phosphate buffer, pH 7.0) in the presence of carbon monoxide until just reduced as determined on a dual wave length spectrophotometer. An aliquot was removed, and the difference spectrum (enzyme with dithionite minus untreated enzyme) was recorded at liquid nitrogen temperatures (---, left scale). To the remainder of the reduced enzyme, excess nitrate was added and the spectrum was recorded after completion of the reaction (---, right scale). The carbon monoxide difference spectrum (reduced enzyme with carbon monoxide minus reduced enzyme) is also shown (---, right scale).
fluoride, and p-hydroxymercuribenzoate inhibited nitrate reduction. Chlorate inhibition was competitive with nitrate. Various metal chelators, such as α,α′-dipyridyl and salicyaldoxime, also inhibited nitrate reductase without affecting cytochrome oxidase activity, implicating an unidentified metal component in nitrate reductase in addition to the participation of a cytochrome.

REFERENCES

Characterization of a Nitrate Reductase from the Chemoautotroph *Nitrobacter agilis*

Patricia A. Straat and Alvin Nason


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