ENZYMATIC ACTIVITY OF NITROSOMONAS EXTRACTS

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Received for publication May 22, 1959

The sole energy-yielding reaction of the autotrophic nitrifying bacterium *Nitrosomonas europaea* involves the conversion of ammonium to nitrite. As early as 1926, Kluyver and Donker postulated that the six electron oxidation proceeds with the intermediary formation of hydroxylamine and hyponitrous acid. Only recently have there been any studies of the biochemical mechanism of chemautotrophic ammonium oxidation.

Hydroxylamine is metabolized by intact cells of this organism and is rapidly and stoichiometrically converted to nitrite (Hofman and Lees, 1953; Engel and Alexander, 1958). Imshenetsky and Ruban (1954, 1956) have reported that autolysates of a *Nitrosomonas* sp. prepared by incubation at 40°C for 24 hr brought about a slow disappearance of ammonium and hydroxylamine in periods of about 5 days. The disappearance of substrate did not result in the formation of appreciable amounts of the nitrite end-product, possibly suggesting the accumulation of some intermediate at the +1 oxidation state of nitrogen although other explanations are possible.

With the availability of relatively large amounts of active cellular material, a reinvestigation of nitrification by enzyme extracts was initiated.

MATERIALS AND METHODS

*N. europaea* was cultured and harvested according to methods described previously (Engel and Alexander, 1958). Cell extracts were prepared by subjecting a 15 to 20 per cent (wet weight) cell suspension to sonic vibration in a Raytheon 10 kc magnetorestrictive oscillator. After a 15-min treatment at temperatures not exceeding 10°C, a deep red, viscous liquid was obtained. The cell debris was removed by centrifugation at 6000 × G for 20 min at a temperature of 3°C

Extracts contained from 1.7 to 3.3 mg N per ml.

Nitrite was determined colorimetrically using α-naphthylamine and sulfanilic acid (Czakay, 1948) and hydroxylamine as the 8-hydroxyquinoline complex (Magee and Burris, 1954). Hydroxylamine and cyanide were neutralized immediately before use. The reduction of methylene blue was measured anaerobically at 660 m&mu; (Umbreit et al., 1957) and 2,4,5-triphenyltetrazolium chloride reduction was determined similarly at 485 m&mu.; Methylene blue was present in a concentration of 0.1 μ mole and the tetrazole at 30 mg. Increases or decreases in optical density of the pyridine nucleotides were measured at 340 m&mu;.

RESULTS

When *N. europaea* extracts prepared by sonic oscillation were incubated with either ammonium or hydroxylamine, nitrite formation could be detected (table 1). The amount of substrate nitrified, however, was small with either of the two compounds and the possibility of residual intact cells or protoplasts could not be entirely eliminated. The net nitrite production by the extract in NH₄ oxidation was 1.0 μg N per ml in 72 hr while, after correcting for controls that estimated chemical decomposition of NH₂OH, there was 1.5 μg NO₂-N per ml formed from hydroxylamine.

The existence of more than one reaction becomes evident if substrate disappearance is measured (table 2). Although no nitrite was detected beyond that observed in controls containing heated enzyme incubated with hydroxylamine, there was a definite enzymatic disappearance of NH₂OH, a reaction abolished by heat denaturation and largely prevented by 0.5 M KCN. The low pH, 7.0, was used to minimize chemical decomposition of the substrate although it was somewhat below the optimum for the metabolism of NH₂OH by the intact organism. The results demonstrate that about one third of...
the substrate had been transformed biologically even without the formation of detectable quantities of nitrite in these shorter periods.

In order to obtain further information on the nature of the NH₂OH-activating system, use was made of the Thunberg technique for determination of dehydrogenase activity (Umbreit et al., 1957). When 0.1 ml of the extract was incubated anaerobically with 10 μmoles NH₂OH and 0.1 μmole methylene blue, the dye was completely reduced to the leuco form in a period of 5 min (figure 1). Boiling the extract or incubation in the presence of 0.01 M potassium cyanide reduced

![Figure 1. Reduction of methylene blue in the presence and absence of potassium cyanide. The Thunberg tubes contained 1.0 ml extract, 0.1 μmole methylene blue, 100 μmoles phosphate, pH 7.0, and 10 μmoles NH₂OH. Cyanide present at a final concentration of 0.01 M.](http://jb.asm.org/)

the activity significantly but did not abolish it. The activity was unaffected by freezing and was present in extracts maintained at 3 C for 2 months.

The use of 2,4,5-triphenyltetrazolium chloride as hydrogen acceptor gave results analogous to methylene blue. The dye was readily reduced, and there was a simultaneous production of formazan as evidenced by the increase in optical density at 485 nm. For example, the optical density increased from 0.097 to 0.680 in 12 hr, whereas the dye was unaltered in the absence of the enzyme. With the boiled enzyme, however, the optical density increased 0.27 in the same time.

To investigate the site of the activity with hydroxylamine, the crude extract was centrifuged at 144,000 × G for 1 hr and resuspended in 0.05 M tris(hydroxymethyl)aminomethane buffer, pH 7.0. The submicroscopic particles containing the cytochromes were packed in a pellet at the bottom of the centrifuge tube and the supernatant liquid

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### TABLE 1

<table>
<thead>
<tr>
<th>Reactants</th>
<th>μg NO₂-N per Ml</th>
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<tbody>
<tr>
<td></td>
<td>0 Hr</td>
</tr>
<tr>
<td>Enzyme + NH₄</td>
<td>0.1</td>
</tr>
<tr>
<td>Heated enzyme + NH₄</td>
<td>0.1</td>
</tr>
<tr>
<td>Enzyme + NH₂OH</td>
<td>0.1</td>
</tr>
<tr>
<td>Heated enzyme + NH₂OH</td>
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</table>

Reaction carried out at 30 C in a system containing 60 μmoles phosphate, pH 8.0; 0.2 ml extract; and 10 μmoles ammonium or 15 μmoles hydroxylamine in a final volume of 3.0 ml. Controls were heated for 15 min at 100 C.

### TABLE 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NH₂OH (μmoles)</th>
<th>Final Nitrite (μmoles)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>0 Hr</td>
<td>1 Hr</td>
</tr>
<tr>
<td>Enzyme + NH₂OH</td>
<td>5.8</td>
<td>5.5</td>
</tr>
<tr>
<td>Heated enzyme + NH₂OH</td>
<td>5.9</td>
<td>5.9</td>
</tr>
<tr>
<td>Enzyme + KCN</td>
<td>5.8</td>
<td>5.7</td>
</tr>
<tr>
<td>NH₂OH</td>
<td>6.1</td>
<td>6.0</td>
</tr>
<tr>
<td>Enzyme</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

Each reaction vessel contained 50 μmoles phosphate, pH 7.0, and a total volume of 5.0 ml. Hydroxylamine was added to a final concentration of 6.0 μmoles. The volume of extract was 0.1 ml and heating was by autoclaving at 15 lb pressure. The cyanide (0.5 M) was neutralized just prior to use, and all initial nitrite analyses were negative.
was a clear, straw color. The sediment was dispersed in 0.05 M tris(hydroxymethyl)amino- methane buffer, pH 7.0, prior to assay. A plot of methylene blue reduction by the nonparticulate fraction with hydroxylamine as substrate is shown in figure 2. A unit of activity was defined as the amount of enzyme necessary to change the optical density 0.001 units per min at a wave length of 660 mµ and at room temperature. Dilution of the enzyme in the supernatant did not cause a reduction or stimulation in the activity; the supernatant contains almost the same activity in both dilutions, 0.01 ml having 49 units and 0.05 ml 250 units. The activity measured is thus proportional to enzyme concentration, and there is no evidence of inhibitors or activators in the extract.

In a sample of the crude extract containing 28,000 units, the supernatant of this high-speed centrifugation contained 30,000 units and the particles 900. The recoverable activity in the oxidation of NH₂OH is therefore almost exclusively present in the supernatant fraction.

Efforts to link the dehydrogenation of ammonium or hydroxylamine with di- or triphosphopyridine nucleotide (DPN, TPN) were undertaken with the reaction mixture containing 10 µmoles of substrate, 100 µmoles of phosphate buffer, pH 8.0, 0.5 ml crude extract, and 300 µg DPN or TPN in a total volume of 3.0 ml. No reduction of either pyridine nucleotide was found in periods up to 11 hr. The extract did cause a slow oxidation of reduced DPN or TPN and also did have a strong catalase activity.

**DISCUSSION**

Cell extracts of *N. europaea* can activate hydroxylamine but show only a limited activity with ammonium as substrate. Similar results have been obtained by Dr. H. Lees (personal communication). In the system described, hydroxylamine disappearance could not be accounted for by the accumulation of nitrite. The hydroxylamine-activating enzyme may be catalyzing a reaction leading to the formation of some intermediate in the nitrification sequence whose nature is as yet unknown, and the conversion of this intermediate to nitrite might then fail to take place because of the lability of the necessary enzyme. Sensitive analytical methods for such compounds are lacking, however, and soluble compounds of this oxidation state of nitrogen tend to decompose rapidly to gaseous products.

It is also possible, however, that the enzyme is not concerned directly in nitrification. Hydroxylamine is known to form oximes and hydroxamic acids and also undergo other enzymatic reactions (Colter and Quastel, 1950). A system of this type would nonetheless be unique for nitrosomonas since the presence in these bacteria of enzymes common to heterotrophs is difficult to demonstrate. The inability to eliminate the reaction completely by cyanide or by boiling may indicate both an enzymatic and a nonenzymatic reaction. More nitrite may be formed in the extracts than is apparent by chemical test, this nitrite being destroyed by reaction with the excess of hydroxylamine (Iwasaki and Mori, 1958). The localization of the enzyme in the nonparticulate portion of the extract may suggest that it is not concerned with nitrification since oxidative phosphorylation and energy storage is usually associated with the particles, and nitrification is the only way by which these
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organisms can generate energy-rich phosphate bonds.

SUMMARY

Extracts of Nitrosomonas europaea slowly oxidize ammonium and rapidly metabolize hydroxylamine. The latter reaction has been coupled to oxidation-reduction dyes. The activity is stable over long periods and is inhibited by cyanide and boiling. The methylene blue activity was entirely recovered in the soluble portion of the cell extract when all particulate components were removed by centrifugation at 144,000 × G.

REFERENCES


