Metabolism under Microaerobic Conditions of Mitochondria from Cowpea Nodules1

STEPHEN RAWSTHORNE2 AND THOMAS A. LARUE*
Boyce Thompson Institute, Tower Road, Ithaca, New York 14853

ABSTRACT

A method is described for isolating mitochondria from nodules of cowpea (Vigna unguiculata [L.] Walp.) under completely anaerobic conditions. The mitochondria were immediately active when incubated anaerobically with substrates, and their respiration rates were higher than mitochondria prepared in air. The mitochondria lacked fumarate reductase and were not inhibited by 5% CO2. When incubated under microaerobic conditions, their respiration could be measured by leghemoglobin spectroscopy. Microaerobic respiration was inhibited approximately 50% by 1 millimolar malonate, and was completely inhibited by cyanide. O2 uptake and the ATP/O ratio declined under microaerobic conditions, and therefore ATP production may be low in the environment of infected nodule cells.

Nodule cells contain large numbers of actively respiring Rhizobium bacteroids and leghemoglobin, which avidly binds O2. As a result, the interior of a legume nodule is microaerobic. In infected cells, the average concentration of free dissolved O2 is only 10 nm, as estimated by leghemoglobin spectroscopy or by a microelectrode (1, 31). Because the Km (O2) for mitochondrial terminal oxidases is considerably greater than this (4), it is questionable if mitochondria in the infected cells have any function, or if they can contribute ATP to the well documented high energy requirement for symbiotic nitrogen fixation.

Seedlings of some species can grow in anaerobic environments. Cells of these plants contain mitochondria, but their morphology is abnormal, and they have weak respiratory activities (32). In the nodule, the mitochondria in infected and uninfected cells appear normal. Their number is higher in infected cells (18), and rather than being distributed with the bacteroids throughout the infected cell, the mitochondria are found only near the cell wall (19).

The presence of mitochondria in the nodule may seem like evidence of aerobic metabolism. This is not so; mitochondria can function in microaerobic metabolism (23). This occurs in species which alternate between aerobic and anaerobic environments (e.g. deep diving fish or whales, swamp worms, molluscs) and in parasitic helminths which pass through aerobic and microaerobic environments in their life cycle. In these species, portions of the tricarboxylic acid cycle 'run backward' in the anaerobic metabolism of carbon compounds. Though subsequent end products differ among species, there is a common anaerobic pathway to succinate. Phosphoenolpyruvate is converted to OAA3 and then malate in the cytosol. The malate passes into the mitochondria where it is converted to fumarate and, in an ATP yielding step, to succinate via fumarate reductase. Mitochondrial fumarate reductase is actually an enhanced activity of the back reaction of succinate dehydrogenase, which is somehow modified during anaerobiosis so that it is less active in the direction of succinate oxidation (27). The reductant for fumarate reductase is NADH which comes from the conversion of another malate to pyruvate by mitochondrial ME. Fumarate reductase activity has not yet been reported in mitochondria from plants.

The mitochondria of Ascaris can produce ATP in microaerobic conditions. They contain Cyt b and c, but Cyt c1, a, and d1 are absent, as is Cyt oxidase. There is only one coupled phosphorylation during electron transport (11).

The vigorous respiration of the nodule produces a high internal CO2 concentration. Mahon, for example, found that pea nodules respired CO2 even when the rhizosphere CO2 concentration was 3% (12). This indicates that the CO2 concentration within the nodule is even higher. As a result, the bicarbonate concentration must also be high in the nodule. Bicarbonate reduces the activity of mitochondria from potato tubers and spinach leaves (6, 16). We therefore determined if mitochondria from the nodule could tolerate high concentrations of CO2.

We described a method for isolating active mitochondria from cowpea nodules (22). The procedure was modified to permit the preparation under completely anaerobic conditions, and subsequent study of the mitochondria under very low pO2. Measurements of ATP formation provided estimates of the P/O ratio under microaerobic conditions.

MATERIALS AND METHODS

Chemicals. Nitrogen gas (prepurified grade) and compressed air were from Union Carbide, Linde Division, New York, NY. CO2 gas (10% CO2 in N2) was from Scott Specialty Gases, Plumsteadville, PA. ATP standards and luciferin/luciferase reagents were from LKB, Gaithersburg, MD. Organic reagents were from Sigma.

Plant Culture. Cowpea (Vigna unguiculata [L.] Walp.) cv California Blackeye, were grown and nodules harvested as described previously (22). For one experiment nodules were obtained from cowpeas planted 20, 27, 33, and 40 d previously. Otherwise, harvests were at 28 to 35 d after planting.

A controlled atmosphere glove box (Labconco, model 50004) was modified by incorporating a 2.5 L cold water bath. The bath was kept at 1°C by heat exchange with a refrigerant circulated from an external Lauda K-21RD cooler. The box and its entry port were flushed with prepurified N2 gas, (~15 μL/L O2) and the gas further purified of O2 by continually circulating the glove.

1 Supported by contract 82ER-12066 from the United States Department of Energy, Division of Biological Energy Sciences.
2 Present address: John Innes Institute, Norwich, NR4 7UH, U.K.
3 Abbreviations: OAA, oxaloacetate; ME, malic enzyme; MDH, malate dehydrogenase.

Received for publication February 13, 1986
box contents through absorption scrubbers (20). Mitochondria. Mitochondria from etiolated hypocotyls were prepared as described previously (22). Mitochondria from nodules were prepared essentially as before (22) except that all steps were conducted in the absence of O₂. Nodules in 1.5 volumes of cold buffer were placed in the entry port of the glove box. The pressure in the entry port was reduced to 50 mm Hg (~7 kPa) for 1 min, and returned to normal with purified N₂. This process was repeated five times but reducing the pressure to only 125 mm Hg (~16 kPa). This caused infiltration of the nodules with the buffer solution. The entry port was flushed for 1 min with O₂ free N₂ from the box, then the nodules were transferred into the glove box where they were gently crushed in plastic bags. After filtering through cheesecloth, the homogenate was placed in screw-capped 50 ml centrifuge tubes which were fitted with an ‘O’-ring seal (Sorvall). After centrifugation, the sample was returned to the glove box. All further manipulations were carried out in the glove box and all centrifugations in sealed tubes.

ATP Determination and ATPase. ATP was determined by the luciferin/luciferase method (14) using an LKB 1250 luminometer. The ATPase activity during purification of leghemoglobin was determined by measuring the rate of decrease in luminescence of a standard mixture. Samples contained 0.45 ml 100 mM Tris acetate (pH 7.75), 2 mM EDTA, 50 μL LKB ATP Monitoring Reagent. 0.5 μM ATP and reactions were started by addition of 5 μl of each column fraction. To ensure that the final purified leghemoglobin was free of ATPase activity, 500 nmol of ATP were incubated with medium A (described later) containing 90 μM leghemoglobin at 28°C. ATP content was determined during incubations of up to 15 min and no significant change in concentrations was recorded.

Leghemoglobin. Unfractionated leghemoglobin was prepared from cowpea and soybean (Glycine max [L.] Merr.) nodules (3). This preparation contained ATPase, so a DEAE-cellulose column-chromatography step (3) was added. After initial fractionation using Sephadex G-75, unfractionated leghemoglobin solution (5 mM in 50 mM phosphate buffer, 1 mM EDTA [pH 7.4]) was dialyzed at 4°C against 10 mM acetic acid (pH 5.2). This solution was fractionated by elution from a DEAE-cellulose column with an acetate gradient. ATPase activity was eluted immediately after starting the gradient and the subsequent fractions of leghemoglobin were pooled and concentrated to 5 mM, and dialyzed against 50 mM phosphate buffer, 1 mM EDTA [pH 7.4] at 4°C. Oxygenation of the hemoprotein was as described by Appleby and Bergersen (3). Leghemoglobin concentrations were determined by difference spectroscopy of the reduced minus oxidized pyridine hemochromes.

O₂ Uptake. O₂ uptake in aerobic conditions (220–20 μM dissolved O₂) was measured with a Gilson 5/6H Oxymeter as before (22). The reaction medium (medium A) contained 0.4 M sucrose, 20 mM Hepes (pH 7.2), 10 mM KCl, 2.5 mM MgCl₂, 4 mM KH₂PO₄, 0.1% BSA (defatted and dialyzed) and known amounts of mitochondrial protein. In some experiments the pH of the reaction medium was adjusted as described previously (22). To equilibrate reaction media with a 5% O₂ gas phase, complete reaction mixtures (excluding mitochondria) were stirred in the O₂ electrode and gently assayed with a mixture (50:50, v/v) of 10% CO₂ in N₂ and compressed air which had been sparged through water to humidify it. Where pH and CO₂ saturation were adjusted, care was taken to ensure that the final pH was correct. At pH 6.4 the solution was aspired with 5% CO₂ until the pH change due to production of HCO₃⁻ and H⁺ was complete. Final adjustment was made with 5 N HCl. At neutral or basic pH values, the pH was adjusted to approximately 0.2 pH units greater than that required and then titrated to the correct pH by aspiration with the 5% CO₂ gas for 2 to 3 min. Dissolved CO₂ concentrations were calculated from the solubility of CO₂ in water at 28°C and were 11.1 μM and 1.58 mM at ambient and 5% CO₂, respectively. The effect of pH on bicarbonate concentration was calculated by the Henderson-Hasselbach equation, from the measured pH and dissolved CO₂ (5). ADP/O ratios were calculated by the method of Estabrook (8).

Microaerobic O₂ uptake was assayed by monitoring the deoxygenation of leghemoglobin solutions at 28°C in sealed cuvettes with no gas phase. The assay solution was medium A which contained 75 to 95 μM leghemoglobin. Additions to the reaction media are detailed in the text where appropriate. Absorbance at 576 and 560 nm was determined at 1 min intervals using a Perkin Elmer Lambda V spectrophotometer; calculations to determine free-dissolved O₂ and the rate of O₂ uptake were as described by Appleby and Bergersen (3).

ATP Extraction. To determine ATP 5 μl aliquots were removed from assay cuvettes with a 10 μl gastight microsyringe (Hamilton, Reno, NV) and immediately quenched into 0.5 ml of ice-cold 1% TCA containing 2 mM EDTA. Samples were cooled on ice for 30 to 40 min and then extracted 3 times with 2 volumes of water-saturated ether. Residual ether was removed by gently aspirating with N₂ gas. Each sample was transferred to a 3 ml glass and glass vial and gently adjusted to 2 ml with 100 mM Tris acetate pH 7.75 containing 2 mM EDTA.

ATP/O Ratio Determinations. The ATP/O ratios during aerobic incubations were obtained by removing aliquots of reaction mixtures from an O₂ electrode chamber containing functioning mitochondria. A 5 μl aliquot was removed 30 s after initiating state 3 respiration (with 30 μM ADP) and a second 5 μl aliquot was removed after a further 45 or 60 s for succinate- or malate-dependent O₂ uptake, respectively. The change in O₂ content during this period was calculated from the rate of O₂ uptake and ratios were calculated as ΔATP/ΔO.

For microaerobic reactions, a 5 μl aliquot was removed from each incubation cuvette 2 to 3 min after adding the mitochondria. Free dissolved O₂ concentrations at this time were measured by leghemoglobin spectroscopy and were between 93 and 148 nM. A second 5 μl aliquot was removed when O₂ uptake became slow (after approximately 20–24 min) when the O₂ concentrations were between 5 and 14 nM. Ratios were calculated as ΔATP divided by twice the O₂ consumed between the two sample times (i.e. ΔO).

Enzymes. Fumarate reductase was measured as described by Kröger and Innerhofer (10).

RESULTS

Nodule mitochondria were isolated under aerobic conditions from plants of different ages (Table I). Their insensitivity to cyanide remained low throughout (4–6% and 3–7% for respiration with malate or succinate, respectively). The highest yield of mitochondrial protein was from young nodules which were still developing, as indicated by their acetylene reduction activity and specific leghemoglobin content (Table I). As plants aged, the amount of mitochondrial protein extracted per gram of nodule declined.

Within the physiological range (6.4–7.5) pH had little effect on malate oxidation rate or oxidative phosphorylation by nodule mitochondria (Table II). At pH 8.0, however, state 3 respiration was inhibited while state 4 respiration was about twice that recorded between 6.4 and 7.5. This indicates some uncoupling of respiration, because the ADP/O ratio was almost halved. Equilibration with 5% CO₂ (to give 1.58 mM dissolved CO₂ and varying HCO₃⁻ concentrations; 1.8–70.8 mM) had only slight effects on oxidation rate and ADP/O ratios, except at basic pH values (Table II). At pH values of 7.5 and 8.0, state 4 respiration was increased, and at the highest pH tested, respiration was uncoupled (ADP/O = 0.9).
Table 1. Respiratory Properties of Aerobically Isolated Nodule Mitochondria in Relation to Age and Development of Nodulated Cowpea Plants

Nodule fresh weights and acetylene reduction activities were determined on four replicate plants of each age. Leghemoglobin content was estimated from the supernatant fraction after pelleting the mitochondria. Reaction media for O2 uptake studies contained 20 mM malate or 10 mM succinate, 0.1 mM ADP and state 3 and state 4 activities were those which followed a third addition of 90 or 95 μM ADP for succinate and malate, respectively.

<table>
<thead>
<tr>
<th>Days After Planting</th>
<th>20</th>
<th>27</th>
<th>33</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodule fresh wt (g plant−1)</td>
<td>2.9</td>
<td>4.8</td>
<td>6.2</td>
<td>6.8</td>
</tr>
<tr>
<td>Acetylene reduction (μmol acetylene g−1 nodule fresh wt h−1)</td>
<td>11.5</td>
<td>18.4</td>
<td>17.4</td>
<td>8.4</td>
</tr>
<tr>
<td>Leghemoglobin content (mg g−1 nodule fresh wt)</td>
<td>1.1</td>
<td>2.4</td>
<td>2.4</td>
<td>1.6</td>
</tr>
<tr>
<td>Extracted mitochondrial protein (μg g−1 nodule fresh wt)</td>
<td>204</td>
<td>79</td>
<td>61</td>
<td>40</td>
</tr>
<tr>
<td>O2 uptake rates: nmol min−1 mg−1 protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Malate</th>
<th>Succinate</th>
</tr>
</thead>
<tbody>
<tr>
<td>state 3</td>
<td>152</td>
<td>220</td>
</tr>
<tr>
<td>state 4</td>
<td>36</td>
<td>96</td>
</tr>
<tr>
<td>RCR</td>
<td>4.2</td>
<td>2.3</td>
</tr>
<tr>
<td>state 3</td>
<td>116</td>
<td>174</td>
</tr>
<tr>
<td>state 4</td>
<td>23</td>
<td>90</td>
</tr>
<tr>
<td>RCR</td>
<td>5.0</td>
<td>1.9</td>
</tr>
<tr>
<td>state 3</td>
<td>95</td>
<td>135</td>
</tr>
<tr>
<td>state 4</td>
<td>18</td>
<td>66</td>
</tr>
<tr>
<td>RCR</td>
<td>5.3</td>
<td>2.1</td>
</tr>
<tr>
<td>state 3</td>
<td>105</td>
<td>180</td>
</tr>
<tr>
<td>state 4</td>
<td>29</td>
<td>84</td>
</tr>
<tr>
<td>RCR</td>
<td>3.6</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Fumarate reductase and succinate dehydrogenase (measured previously [22]) in mitochondria from nodules and hypocotyls had similar activities in both preparations, and the ratios of their activities were identical (Table III).

When nodule mitochondria isolated in the absence of O2 were incubated in an O2 electrode chamber with aerobic concentrations of O2 and respiratory substrates and cofactors, O2 uptake was immediate and showed a pattern similar to that recorded previously for mitochondria prepared aerobically [22]. Microaerobic preparations had higher rates of substrate oxidation compared to mitochondria prepared in air (Table IV). State 3 and state 4 activities were 1.5- to 1.6-fold higher for succinate-dependent respiration while oxidation of malate was increased slightly less. When malonate was added to nodule mitochondria during state 4 respiration with 10 mM succinate, O2 uptake was inhibited. A Dixon plot for malonate concentrations between 0.5 and 3.0 mM gave an 'apparent' Km of 1 mM.

To determine the effects of very low O2 concentrations (150–5 nm) on O2 uptake, mitochondria prepared microaerobically from nodules were incubated in complete reaction media containing leghemoglobin. Similar experiments were carried out on aerobically isolated hypocotyl mitochondria. Glutamate (10 mM) was included with malate in studies of microaerobic malate oxidation as it prevented a gradual decline in state 3 respiration of nodule mitochondria during prolonged aerobic incubations in the presence of a large excess of ADP (data not shown). The decline was probably due to accumulation of oxaloacetic acid (6). Glutamate may partly alleviate this by promoting activity of glutamate:OAA transaminase (9) and so removing the OAA. Oxidation of glutamate alone was slight by either mitochondrial preparation (data not shown). Addition of glutamate was therefore necessary to avoid confounding the effects of O2 concentration on malate oxidation with the inhibitory effects of continuous malate oxidation per se.

O2 concentrations of 200 nm or less were limiting to state 3 respiration of nodule or hypocotyl mitochondria during oxidation of malate or succinate (Figs. 1, 2). In contrast, state 4 respiration rates with malate were less affected by decreasing O2 concentration and so the degree of apparent respiratory control decreased (Fig. 1). State 4 oxidation of succinate was faster than that of malate and showed greater decreases in activity as O2 concentration declined (Fig. 2). At limiting O2 concentrations, O2 uptake with either organic acid was always greater for mitochondria from nodules than those from hypocotyls.

The apparent Km (O2) of the terminal oxidase of the intact mitochondrial system was derived from double reciprocal plots of rapid state 3 respiration during the decline in O2 concentration (i.e. before O2 uptake in the presence of excess ADP showed significant slowing and resemblance of state 4 activity). The Km (O2) for both the nodule and hypocotyl oxidase system was approximately 100 nm (Fig. 3).

O2 uptake by nodule mitochondria under microaerobic conditions was inhibited by KCN. Addition of 0.2 mM KCN at 40 mM O2 inhibited succinate oxidation by 94%. Addition of 1 mM malonate to nodule mitochondria oxidizing succinate under microaerobic conditions approximated halved the rate of O2 uptake.

The ADP/O ratio, determined aerobically, was compared to ATP/O ratios measured during aerobic or microaerobic oxidation of malate or succinate. At saturating O2 concentrations (220–20 μM) the ADP/O and ATP/O ratios were similar for nodule mitochondria (Table V). While nodule mitochondria oxidized malate or succinate and O2 concentrations declined

Table II. Effect of pH and a 5% CO2 in Air Atmosphere on Malate Oxidation by Nodule Mitochondria

Parameters of malate oxidation are given for the second addition of 90 μM ADP to reaction mixtures containing 20 mM malate, 0.1 mM ATP and mitochondria. Bicarbonate concentrations were derived from calculations based upon the solubility of CO2 in water at 28°C and the subsequent pH-dependent equilibration with HCO3− in solution.

<table>
<thead>
<tr>
<th>pH</th>
<th>HCO3− calc</th>
<th>O2 uptake state 3</th>
<th>O2 uptake state 4</th>
<th>ADP/O</th>
<th>HCO3− calc</th>
<th>O2 uptake state 3</th>
<th>O2 uptake state 4</th>
<th>ADP/O</th>
</tr>
</thead>
<tbody>
<tr>
<td>mm</td>
<td>mmol/min-mg protein ratio</td>
<td>mm</td>
<td>mmol/min-mg protein ratio</td>
<td></td>
<td>mm</td>
<td>mmol/min-mg protein ratio</td>
<td>mm</td>
<td>mmol/min-mg protein ratio</td>
</tr>
</tbody>
</table>
chondria were comprise most trations were ratios of O\textsubscript{2}/O\textsubscript{2} ratios of 1.3 and 0.5, respectively. Corresponding ADP/O ratios of hypocotyl mitochondria under saturating O\textsubscript{2} concentrations were 2.4 and 1.6, respectively (22).

**DISCUSSION**

The yield of mitochondria decreases with increasing plant age. The high yield from young developing nodules may reflect a high energy demand for nodule growth. Alternatively, older nodules may contain factors that damage mitochondria during the isolation procedure.

Other experiments described here used plants 28 to 35 d old, before nodule senescence was significant. Although the nodule is highly differentiated we are confident that most of the mitochondria were derived from the infected zone, where the O\textsubscript{2} is very low. In a large soybean nodule the bacteroid zone will comprise most of the tissue, and within this zone the uninfected cells are about twice as numerous as infected cells (24). It is estimated that infected cells may have 3 to 4 times more mitochondria per cell than uninfected cells (18). Consequently, a preparation of mitochondria from the nodules should contain more mitochondria from infected than uninfected cells, assuming that extractability is uniform throughout the tissue. Because the nodules used in most of these experiments were large, the majority of the tissue will have been from the infected zone.

Malate oxidation under aerobic conditions was barely affected by high concentrations of dissolved CO\textsubscript{2} and HCO\textsubscript{3}\textsuperscript{-} except at high pH. There was an apparent uncoupling of respiration, loss of respiratory control, and decline in ADP/O ratio at high pH values (Table II). This effect was reported for mitochondria from pea leaves incubated at pH 7.4 versus 6.8 (15) but the mechanism remains speculative. Effects of pH on the rate of malate oxidation by mitochondria from spinach leaves and potato tubers have been ascribed to the compound effects of pH and HCO\textsubscript{3}\textsuperscript{-} concentration on NAD\textsuperscript{+}-ME (6, 16). Mitochondria from cowpea nodules have low NAD\textsuperscript{+}-ME activity (22) and their malate oxidation is dependent on MDH activity. The activity of MDH must therefore be unaffected by either pH or high dissolved CO\textsubscript{2} and HCO\textsubscript{3}\textsuperscript{-} concentrations. Thus, it appears that nodule mitochondria are tolerant to their high CO\textsubscript{2} environment.

Malonate, a strong inhibitor of succinate dehydrogenase, inhibited succinate oxidation with an apparent K\textsubscript{i} of 1 mM. Soybean nodules have a high malonate content (29) but whether it is free in the cytosol is unknown. If malonate is in the infected cells it is likely to be free because infected cells of determinant type nodules are nonvacuolate (28). Bacteroids from soybean nodules can metabolize exogenous malonate, and it does not inhibit their use of succinate (33). Thus, the possibility of malonate being in the infected cells cannot be excluded. If this is so, operation of the tricarboxylic acid cycle in the mitochondria might be limited.

In some facultative organisms, the oxidative activity of succinate dehydrogenase is depressed under microaerobic conditions, and its reverse activity of fumarate reduction increased (23, 27). The specific activities of succinate dehydrogenase in mitochondria isolated from the nodule and from the hypocotyl were identical (22; Table III). The rate of fumarate reduction was similar in both preparations, as was the ratio of fumarate reduction/succinate oxidation. This constant ratio indicates that the activity detected is not fumarate reductase, but only the back

---

**Table III. Succinate Dehydrogenase and Fumarate Reductase Activities of Mitochondria from Cowpea Nodules and Etiolated Hypocotyls**

Assays of each enzyme were performed on two separate preparations from each tissue and the mean is presented in the table.

<table>
<thead>
<tr>
<th>Source of Mitochondria</th>
<th>Enzyme Activity</th>
<th>Succinate Dehydrogenase</th>
<th>Fumarate Reductase</th>
<th>Succinate Dehydrogenase/Fumarate Reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodule</td>
<td>1178</td>
<td>98</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>Hypocotyl</td>
<td>1180</td>
<td>109</td>
<td>10.8</td>
<td></td>
</tr>
</tbody>
</table>

**Table IV. Aerobic Oxidative Activities of Nodule Mitochondria Prepared Aerobically or Anaerobically**

Reaction media, substrate concentrations, and recording of respiration rates were as described in Table I. Values within the table represent the mean ± SE of at least five separate aerobic or anaerobic preparations performed during a 6 week period.

<table>
<thead>
<tr>
<th>Respiratory State</th>
<th>Malate Preparation</th>
<th>Succinate Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aerobic</td>
<td>Anaerobic</td>
</tr>
<tr>
<td></td>
<td>Aerobic</td>
<td>Anaerobic</td>
</tr>
<tr>
<td></td>
<td>nmol O\textsubscript{2}/min-mg protein</td>
<td>97 ± 6</td>
</tr>
<tr>
<td></td>
<td>94 ± 1</td>
<td>16 ± 1</td>
</tr>
</tbody>
</table>

from 150 to 5 mM, the efficiency of phosphorylation (ATP/O) was at least halved compared to aerobic conditions (Table V). In an unreplicated experiment, oxidation of malate or succinate by hypocotyl mitochondria under conditions of limiting O\textsubscript{2} gave ATP/O ratios of 1.3 and 0.5, respectively. Corresponding ADP/O ratios of hypocotyl mitochondria under saturating O\textsubscript{2} concentrations were 2.4 and 1.6, respectively (22).
reaction of undepressed succinate dehydrogenase. In facultative animals, the reductant for mitochondrial fumarate reductase is NADH from mitochondrial NADH-ME (23). We have noted (22) that the mitochondria isolated from nodules have only a low activity of NADH-ME. We conclude that, unlike the mitochondria in facultative animals, the mitochondria of the nodule do not use fumarate reductase for ATP synthesis.

Microaerobic oxidation rates of organic acids in the presence of ADP were considerably greater for mitochondria from nodules than from hypocotyls even though their $K_m$ ($O_2$) were identical. This is in contrast to aerobic oxidation where hypocotyl mitochondria were superior at oxidizing either malate or succinate and particularly the former substrate (these data and [22]). Under aerobic conditions where Cyt oxidase is saturated, it is probable that oxidation is limited by the rate of substrate uptake or metabolism and not by the capacity for electron transport. If O$_2$ becomes limiting, however, the rate of O$_2$ uptake may be determined primarily by the amount of Cyt oxidase ($i.e.$ the capacity for electron flux). Nodule mitochondria had almost twice the Cyt oxidase activity of hypocotyl mitochondria, 7.9 and 4.2 $\mu$mol min$^{-1}$ mg$^{-1}$ protein, respectively (22). This may explain why O$_2$ uptake was greater by the former under limiting O$_2$ conditions.

Despite the higher Cyt oxidase content in mitochondria from nodules, their respiration at 10 nm O$_2$ was only 8 to 9% of that measured in air, with both malate and succinate (Figs. 1, 2). The measurements of microaerobic respiration clearly show that O$_2$ uptake by nodule mitochondria would be limited at 10 nm O$_2$, the concentration which prevails in the nodule (1, 31).

Not only would the O$_2$ flux be limited but the efficiency of coupling of O$_2$-dependent electron transport to ATP synthesis would also be less than in the presence of saturating O$_2$. The decrease in ATP/O ratio with limiting O$_2$ concentrations is clear (Table V) and even in the presence of excess ADP, the stimulation of O$_2$ uptake progressively decreased as O$_2$ concentration declined (Figs. 1, 2). The mechanism behind this decline in the ATP/O ratio was not investigated. It may relate to a decline in the proton motive force as substrate-driven electron transport, and hence proton efflux, became limited as the O$_2$ concentration declined (7).

In this investigation of microaerobic function of mitochondria we have derived an integrated ATP/O ratio which was based on O$_2$ uptake from an O$_2$ concentration slightly greater than the $K_m$ (O$_2$) to as low as 5 nm. We did not determine what the ATP/O ratio would be if the pO$_2$ was maintained at 10 nm. At 10 nm O$_2$ ATP synthesis is likely to be slight because ADP caused no stimulation of O$_2$ uptake when mitochondria from either nodules or hypocotyls were oxidizing malate or succinate (Figs. 1, 2). Because this is the average free-dissolved O$_2$ concentration in the nodule (1, 31) there are serious implications for mitochondrial function, at least in terms of ATP generation.
Electron micrographs of nodules show mitochondria almost exclusively at the periphery of infected cells (19) and, in some cases, grouped at the periphery adjacent to air spaces. These observations suggest that during development of an infected cell the mitochondria move outward in response to an O2 gradient. Such responses to O2 gradients have been reported for mitochondria in secretory cells of insect larvae (30).

A model based on two-dimensional analysis of O2 flux and free-dissolved O2 concentration within an infected nodule cell indicated that an O2 concentration gradient may exist (25). The model predicts O2 concentrations of 5 nm at the center and 26 nm at the cell periphery. At the higher of these O2 concentrations, O2 uptake is slightly stimulated by ADP (Fig. 1) and so ATP synthesis is more likely than at 10 nm O2, despite an ATP/O ratio which may be less than optimal. Nevertheless, without detailed knowledge of the exact O2 environment which nodule mitochondria experience, either in infected or uninfected cells, we have to conclude that both O2 uptake and the ATP/O ratio are likely to be much lower than in aerial tissues.

The carbohydrate cost to the plant of symbiotic fixation has been often estimated (cf. reviews [13, 17, 21]). The consumption of photosynthate, though calculated in different ways, always seems greater than required for the known ATP requirements of nitrogen fixation. In estimating how much energy is obtained from the photosynthate, the P/O ratio in nodules has been assumed to be 3 by Neves (17) and Mahon (13) and 2 by Phillips (21). It is probable that these estimates were too high. That the interior of nodules has a very low Po2 has long been known, but the implications of this for intermediary metabolism and energy production have been overlooked. Because many bacteroids lack Cyt AA2 (2), their P/O ratio is unlikely to exceed 2. Experiments reported here indicate that mitochondria in the infected cell will contribute little if any ATP from oxidative phosphorylation. This is a probable contributing factor for the high photosynthetic cost of symbiotic nitrogen fixation.

LITERATURE CITED