Nitrogen Fixation by Cultures and Cell-free Extracts of Mycobacterium flavum 301

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SUMMARY

Growth, nitrogen fixation and acetylene reduction by Mycobacterium flavum 301 (NCIB 10,071) were increased with sodium lactate, pyruvate, gluconate or succinate as compared with ethanol, a recommended substrate. Yeast extract could be replaced with (NH₄)₂SO₄; in continuous culture a source of fixed nitrogen could be omitted altogether. Growth, nitrogen fixation and acetylene reduction all increased at lowered PO₂ values. Wholly anaerobic conditions did not support growth. Nitrogen fixation was confirmed isotopically.

Cell-free extracts performed the following reductions: N₂ to NH₃, H⁺ to H₂, C₂H₂ to C₂H₄, HCN to CH₄, CH₃NC to CH₄ + C₂H₂, KCN to CH₄, C₂H₄ to C₂H₂, Mg²⁺, Na₂S₂O₄, and anaerobic conditions during preparation and assay of extracts were required. 3·5 mole ATP were hydrolysed to release 1 mole H₂. Pyruvate, α-ketobutyrate, α-ketoglutarate, succinate, glucose and glucose-6-phosphate did not replace dithionite. ADP, AMP or high concentrations of ATP inhibited reduction. Activity was associated with a particle which sedimented at 145,000 g over 3½ hr. The nitrogenase system of M. flavum thus resembles the particulate system of Azotobacter, rather than the soluble pyruvate-utilizing system of Clostridium pasteurianum.

INTRODUCTION

Nitrogen-fixing members of the genus Mycobacterium were first isolated from Russian turf-podzol soils by Fedorov & Kalininskaya (1961a). They may be the important nitrogen fixers in these soils because Azotobacter is scarce in such relatively acid environments and these mycobacteria are more acid-tolerant than most Azotobacter species (Fedorov & Kalininskaya, 1959, 1961a; L'vov, 1963). Three nitrogen-fixing species of mycobacteria have been reported: Mycobacterium flavum 301, M. roseo-album 368, Mycobacterium sp. 571.

Fedorov & Kalininskaya (1959, 1960; 1961a, b) originally grew nitrogen-fixing mycobacteria in mixed cultures with other bacteria with which they are probably associated in the soil (Kalininskaya, 1967a, b, c). The associated bacteria stimulated growth and were thought to degrade carbon substrates to an accessible form as well as supply supplementary growth factors, including vitamins.

The particular species used in the present work, Mycobacterium flavum 301 (Fedorov & Kalininskaya, 1960; 1961a, b), is unable to utilize carbohydrates but grows on ethanol or organic acids. Fedorov & Kalininskaya (1961c) added yeast extract as a source of growth factors to media based on such carbon sources, in order to maintain the organism in pure culture. Il'ina (1966a, b; 1967a, b; 1968) investigated the metal ion requirements of M. flavum 301. Molybdenum was specifically required for nitrogen
fixation but at a concentration many times less than those needed by other nitrogen-fixing micro-organisms. This feature may be another reflexion of their acid turf-podzol habitat, which is notably low in available molybdenum (Il'ina, 1966a; Mishustin & Krylova, 1965). Cobalt was also specifically required for nitrogen fixation; utilization of ammonium chloride nitrogen did not depend on molybdenum or cobalt. Other trace elements (Cu, Fe, B, Mn, Zn) stimulated nitrogen fixation non-specifically. In this paper cultural procedures for improving growth and nitrogen fixation by *M. flavum* 301 are reported, together with the extraction and some properties of a cell-free nitrogen-fixing system; a preliminary report has appeared (Biggin & Postgate, 1969).

**METHODS**

*Organism and medium.* *Mycobacterium flavum* strain 301 was kindly supplied by Dr T. A. Kalininskaya: a culture is now held by the National Collection of Industrial Bacteria with the catalogue number NCIB 10,071. The organism grew well at 30° on nutrient agar (Oxoid Ltd.) to form homogeneous yellow colonies. For nitrogen-fixation tests a medium based on one prescribed by Dr Kalininskaya (personal communication) was used: K₂HPO₄, 1.67 g.; KH₂PO₄, 0.87 g.; MgSO₄.7H₂O, 0.29 g.; CaCl₂, 0.07 g.; NaCl, 0.48 g.; FeCl₃, 6H₂O, 0.01 g.; Na₂MoO₄.2H₂O, 0.005 g.; ZnSO₄.7H₂O, 0.0002 g.; MnSO₄.4H₂O, 0.003 g.; H₃BO₃, 0.005 g.; CoSO₄.7H₂O, 2 µg.; biotin, 20 µg.; yeast extract (Oxoid Ltd.), 0.08 g.; carbon source, 4 g.; distilled water, 1 l, pH 6.8.

*Culture at various oxygen tensions.* To determine the optimum pO₂ for growth and nitrogen fixation, 50 ml. portions of medium in 250 ml. conical flasks were inoculated with 0.4 ml. of a 1/10 dilution in 11-85% NaCl of an actively growing culture. The flasks were flushed with high purity nitrogen and the cotton-wool plugs replaced by sterile 'Suba-seal' rubber stoppers in a stream of nitrogen. Different pO₂ values were established by replacing nitrogen with oxygen by using a syringe inserted through the rubber seal. The cultures were shaken in an orbital shaker at 150 rev./min. at 30° up to 16 days. Samples of the culture fluid were removed aseptically at 2 to 3 day intervals by opening the flask; after sampling the rubber seal was replaced and the appropriate pO₂ re-established. The purity of each culture was tested by plating on nutrient agar at the end of the experiment.

*Growth measurements.* Extinctions in a 1 cm. cell at 540 mμ were measured in an EEL Spectra spectrophotometer and converted to dry weight organisms/ml. by using a standard curve.

*Culture under ¹⁵N.* Incorporation of ¹⁵N by growing cultures was tested by Miss S. Hill in this laboratory by using conventional methods (Burris & Wilson, 1957). Cultures were shaken in 100 ml. of Kalininskaya's medium containing yeast extract and sodium lactate (4 g./l.) in a 250 ml. flask under an atmosphere with pO₂ = 0.1, pN₂ = 0.2, pAr = 0.7 atm. 7 ml. of 95% enriched ¹⁵N₂ was injected to give a final enrichment of 15 atom % excess. pO₂ was monitored during growth by gas chromatography and oxygen injected aseptically every 1 to 2 days as necessary to maintain a pO₂ of 0.08 to 0.10. Two controls were included: one contained acidified medium, to take up residual ¹⁵NH₃ from the isotopic gas sample; the other was a culture of a non-nitrogen-fixing organism, *Escherichia coli*. After Kjeldahl digestion and Markham distillation of 12-day cultures, the ¹⁵N enrichment of samples was determined by mass spectroscopy by Dr C. W. Crane (Queen Elizabeth Hospital, Birmingham 15).
Continuous culture. Mycobacterium flavum 301 was grown continuously in an apparatus of the type described by Baker (1968) in Kalininskaya’s medium with 4 g. sodium lactate/l. under N₂-air mixtures which flowed over the culture at 490 ml./min.; dilution rate, 0.05 hr⁻¹; temperature, 30°. The culture volume was 580 to 670 ml. according to stirring rate: a low stirring rate (250 rev./min.), which created negligible vortex, provided conditions of relatively low aeration (oxygen solution rate was 0.6 mmole O₂/l./hr); a high stirring rate (1000 rev./min.) resulted in a vortex extending to the bottom of the culture vessel with copious upward bubbling through the medium (oxygen solution rate, 30.4 mmole O₂/l./hr). Oxygen solution rates were measured by sulphite oxidation (Elsworth, Williams & Harris-Smith, 1957) with the vessel open to the atmosphere.

Large scale culture. Large Pyrex bottles (20 l.) containing 18 l. of Kalininskaya’s medium with 4 g. sodium lactate/l. were inoculated with about 1 l. of culture from either the overflow of a continuous culture or a ‘seed’ batch culture in the same medium. During growth the cultures were sparged with air+nitrogen mixture (pO₂ = 0.05 atm.) at approx. 2.5 l./min. and maintained at 30°. After 2 to 3 days, when the yield exceeded equiv. 0.33 mg. dry wt organisms/ml., the bacteria were harvested by batch centrifugation for 8 min. at 40,000 g in an MSE ‘High Speed 18’ centrifuge. They were washed in 0.025 M-tris HCl buffer at pH 7.2, and the cell paste stored as pellets in liquid nitrogen.

Acetylene reduction by cultures. Batch cultures (25 ml. in 250 ml. volumetric flasks) were inoculated with 0.2 ml. of a 1/10 dilution in 0.85% NaCl of an actively growing culture and a Suba-seal inserted above the cotton-wool plug. Acetylene was injected to 0.02 atm. and samples were removed for vapour phase chromatography every 2 to 3 days during incubation. Samples from a continuous culture (D = 0.05 hr⁻¹, low stirring rate, pO₂ over culture 0.1 atm., population equiv. 0.26 mg. dry wt organism/ml.) were tested with 0.04 atm. acetylene in a similar way except that 10 ml. portions were incubated for up to 2 hr in sealed 25 ml. conical flasks flushed with nitrogen. Atmospheres of different pO₂ value were obtained by injecting O₂ after an equivalent volume of N₂ had been removed with a syringe.

Preparations of cell extracts. For each experiment, frozen cell paste was thawed at room temperature under argon, 1 to 2 vol. 0.025 M-tris HCl buffer (pH 7.2) was added and the whole stirred to an even consistency. Batches of cell suspension (15 ml.), cooled in ice, were sonicated under a stream of argon by using a Dawe stainless steel Soni-probe at 8 A for 1 min. The sonicate was centrifuged at 38,000 g for 30 min. under argon and the yellow supernatant solution (4 to 13 mg. protein/ml.) stored in ice under argon for not more than 1 hr before use.

Analyses. Nitrogen contents of growing cultures were determined by Kjeldahl digestion followed by Nesslerization (Umbreit, Burris & Stauffer, 1964), and fixation calculated by difference from acid-killed controls. Nitrogen fixed by cell-free extracts was measured as ammonia (Dilworth, Subramanian, Munson & Burris, 1965). Protein was estimated by the biuret method, as modified by Gornall, Bardawill & David (1949).

Gas samples were analysed for hydrocarbons by using a Pye series 104 gas chromatograph with a 5 ft. (152 cm.) Porapak R column at 45° in a stream of N₂ (50 ml./min.) with a flame-ionization detector. The assay system for cell-free reduction of nitrogen, acetylene, cyanide or methyl isocyanide was based on that of Bulen, Burns & Le
Comte (1965): 12.5 µmole tris HCl buffer (pH 7.2), 5 µmole MgCl₂, 20 µmole Na₂S₂O₄ and an ATP-generating system (2.5 µmole ATP, 20 µmole creatine phosphate, 0.2 mg. creatine kinase) in a final volume of 1 ml. were contained in 7.5 ml. Suba-sealed ampoules. Reaction was at 30° under argon with shaking, and was started by adding extract (equiv. 2.5 to 5.6 mg. protein). Gas samples for vapour phase chromatography were removed at 15, 30 and 45 min. by using a syringe inserted through the Suba seal. When the ATP-generating system was omitted the MgCl₂ was decreased to 2 µmole and ATP increased to 4 µmole. Gas samples were then removed at 6 and 12 min. because the reaction rate fell sharply after 15 min.

ATP-activated hydrogen evolution and reductant-dependent ATP-ase were measured in Warburg vessels (nominally 15 ml.) with a rubber-capped side arm, containing cell-free extract (19.1 to 22.1 mg. protein) in the main compartment; 37.5 µmole tris HCl buffer (pH 7.2), 12.5 µmole MgCl₂, 7.5 µmole ATP, 50 µmole creatine phosphate, 0.5 mg. creatine kinase and 50 pmole Na₂S₂O₄ in the side arm; 0.1 ml. 40% KOH in the centre well; Vᵣ = 2.5 ml. The flasks were flushed with argon for 10 min., extract added to the main compartment and dithionite to the side arm via the rubber cap by using a syringe, and then flushing was continued for a further 5 min. The reaction was started by tipping the contents of the side arm and was at 30° with shaking. After 25 min., while the reaction rate was still linear, it was stopped by adding 0.1 ml. 40% KOH, and inorganic phosphate determined by the method of Taussky & Shoor (1953).

Conventional hydrogenase activity was detected as follows: whole organisms equivalent to 13.8 mg. dry wt organism, or cell-free extract (2-6 to 7.8 mg. protein), were added to an equal volume of 0.07 M-potassium phosphate buffer (pH 7) + 0.1 mM-dye in Thunberg tubes containing H₂. Dye reduction rates were compared with controls under argon.

Reagents. Chemicals used in this work were the best grade commercially available. Isotopic nitrogen as 95% enriched ¹⁵N₂ gas was obtained from M. W. Hardy & Co., Mercantile Ltd., London, E.C. 2. Biochemicals were obtained from Sigma (London) Chemical Co., London, S.W. 6. ATP, ADP and AMP (neutralized 0.1 M solutions) and creatine kinase (2 mg./ml. 0.1 0.025 M-tris HCl buffer, pH 7.2) were stored at −20°. ADP contained approximately 20% AMP as indicated by thin layer chromatography. 0.2 M-Na₂S₂O₄ was prepared as required and stored for not more than 2 hr under argon. Methyl isocyanide was prepared by Dr R. L. Richards following the procedure of Shaw & Pritchard (1966), stored at −70° in a solid-CO₂ cabinet, and solutions in water were prepared as required and discarded after 4 hr. Purified acetylene (99.6% minimum) was obtained from Matheson Chemical Co., New Jersey, U.S.A., or produced from calcium carbide and water.

RESULTS

Effect of pO₂ on growth and nitrogen fixation. The data in Table 1 illustrate the conclusion from many samplings: that a pO₂ of 0.1 atm. was optimal for growth and nitrogen fixation in batch culture. The rates of increase of both parameters were double those at normal atmospheric composition. The slight growth and nitrogen fixation which occurred at a pO₂ of zero was attributed to failure to exclude oxygen completely, since they were eliminated in other tests when oxygen was rigorously excluded by the use of a pyrogallol plug.
Nitrogen fixation by Mycobacterium flavum 301

Table 2 illustrates comparable data for a continuous culture: steady state values for growth and nitrogen fixation increased when the pO$_2$ of the gas flowing over the culture was decreased from 0.1 to 0.5 atm., provided stirring rate was increased.

These observations indicated that a pO$_2$ value below atmospheric favoured growth and nitrogen fixation by Mycobacterium flavum 301, though wholly anaerobic conditions did not support growth. The difference in the optimal pO$_2$ between batch and continuous culture is discussed later. For the rest of the work reported here shake-flask cultures were routinely grown at pO$_2$ = 0.1 atm. and 20-l. batch cultures were sparged with pO$_2$ = 0.05 atm. air + nitrogen mixtures.

Table 1. Effect of pO$_2$ on Mycobacterium flavum 301 in batch culture

Cultures were shaken at 30°, with lactate medium (see text) under the atmospheres indicated, and sampled at intervals. Twelve-day values are quoted as typical of many.

<table>
<thead>
<tr>
<th>Organism</th>
<th>pO$_2$ (atm.)</th>
<th>Organism</th>
<th>Nitrogen fixed (µg N/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>concentration</td>
<td>mg. dry wt/ml.)</td>
<td>fixed</td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>0.10*</td>
<td>10*</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>0.40</td>
<td>9.9</td>
<td></td>
</tr>
<tr>
<td>0.02</td>
<td>0.75</td>
<td>17.8</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>1.15</td>
<td>31.4</td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>1.37</td>
<td>60.0</td>
<td></td>
</tr>
<tr>
<td>0.20</td>
<td>0.63</td>
<td>29.6</td>
<td></td>
</tr>
</tbody>
</table>

* Marginal growth probably due to incomplete anaerobiosis (see text).

Table 2. Effect of pO$_2$ and stirring rate on Mycobacterium flavum 301 in continuous culture

Steady states were established at D = 0.052 ± 0.003 hr$^{-1}$ in lactate medium at 30° under air + N$_2$ mixture at 'low' and 'high' stirring rates (see text).

<table>
<thead>
<tr>
<th>Stirring rate</th>
<th>pO$_2$ (atm.)</th>
<th>Organism concentration (mg. dry wt/ml.)</th>
<th>Nitrogen fixed (µg N/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>0.1</td>
<td>0.26</td>
<td>5.7</td>
</tr>
<tr>
<td>Low</td>
<td>0.05</td>
<td>0.10</td>
<td>0.9</td>
</tr>
<tr>
<td>High</td>
<td>0.05</td>
<td>0.64</td>
<td>12.2</td>
</tr>
</tbody>
</table>

Isotopic nitrogen fixation. The analytical figures from the above experiments leave little doubt that true nitrogen fixation had occurred, not merely scavenging of atmospheric fixed-nitrogen. Nevertheless cultures were tested for incorporation of isotopic nitrogen. Twelve-day-old shake-flask cultures of M. flavum 301, grown in an atmosphere with 15 atom % $^{15}$N$_2$ excess were enriched 9.216 atom % $^{15}$N excess compared with enrichments of 0.003 and 0.004 atom % $^{15}$N excess for acidified medium and Escherichia coli controls respectively.

Acetylene reduction by M. flavum 301. All nitrogen-fixing organisms and extracts so far studied reduce acetylene to ethylene, and this reaction has become a rapid and sensitive assay for nitrogenase activity (Dilworth, 1966; Schöllhorn & Burris, 1966, 1967). Preliminary tests in batch cultures indicated that M. flavum 301 cultures reduced
acetylene. Cultures into which 0.02 atm. acetylene were injected were therefore included in our investigations.

**Effect of various carbon sources in batch culture.** Several different carbon sources supported greater growth, nitrogen fixation and acetylene reduction than did ethanol, the substrate recommended by Dr Kalininskaya (Table 3). Lactate was chosen for routine use because it was readily available and could be autoclaved.

Table 3. Effect of various carbon sources on growth, nitrogen fixation and acetylene reduction by *Mycobacterium flavum 301* in batch cultures

Cultures were shaken at 30° with 0.4% (w/v) substrate (anions as the sodium salt) in sealed flasks with the atmospheres indicated. The first two columns record data from the same cultures, the third from a parallel series with 0.02 atm. C₂H₂ above the culture. Data are representative of several samplings.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Growth (mg. dry wt/ml.)</th>
<th>Nitrogen fixed (μg. N/ml.)</th>
<th>Ethylene produced (μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate</td>
<td>0.56</td>
<td>25.9</td>
<td>23.8</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.46</td>
<td>16.8</td>
<td>38.1</td>
</tr>
<tr>
<td>Gluconate</td>
<td>0.45</td>
<td>19.2</td>
<td>4.6</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.42</td>
<td>15.4</td>
<td>19.6</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.29</td>
<td>11.5</td>
<td>15.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Growth (mg. dry wt/ml.)</th>
<th>Nitrogen fixed (μg. N/ml.)</th>
<th>Ethylene produced (μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate</td>
<td>0.16</td>
<td>2.7</td>
<td>0.04</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.17</td>
<td>9.9</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Table 4. Growth, nitrogen fixation and acetylene reduction by *Mycobacterium flavum 301* in batch culture with (NH₄)₂SO₄

Duplicate cultures were grown at pO₂ = 0.1 with 4 g. carbon source/l. and with (NH₄)₂SO₄ 40 mg./l. replacing yeast extract 80 mg./l. in Kalininskaya's medium. Acetylene was at 0.02 atm. and gas samples were removed at intervals. Extinction coefficients and nitrogen contents were measured at the end of the experiment.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Growth (mg. dry wt/ml.)</th>
<th>Nitrogen fixed (μg. N/ml.)</th>
<th>Ethylene produced (μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate</td>
<td>0.72</td>
<td>26.1</td>
<td>26.8</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.38</td>
<td>19.7</td>
<td>34.6</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.22</td>
<td>12.5</td>
<td>16.8</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.38</td>
<td>13.9</td>
<td>5.1</td>
</tr>
</tbody>
</table>

**Requirement for yeast extract.** Though yeast extract is often used in media for nitrogen-fixing mycobacteria (L’vov, 1963), (NH₄)₂SO₄ at equivalent nitrogen concentration supported growth, nitrogen fixation and acetylene reduction in batch cultures with a variety of carbon substrates (Table 4). This observation confirmed the personal communication from Dr Kalininskaya that yeast extract was not essential. In
Nitrogen fixation by Mycobacterium flavum 301

In continuous culture a source of fixed nitrogen could be dispensed with altogether: a steady state at $D = 0.05$ hr$^{-1}$, $pO_2 = 0.05$ atm., high stirring rate, was maintained in a medium containing 80 mg. yeast extract/l. for eight days. When the medium was changed to one containing neither yeast extract nor $(NH_4)_2SO_4$, the steady state was maintained for a further 9 days; growth and nitrogen fixation continued undiminished when the dilution rate was increased to 0.1 hr$^{-1}$ for another 12 days.

Effect of $pO_2$ on acetylene reduction in short-term experiments. The conclusion that a low $pO_2$ favoured growth and nitrogen fixation was tested by using the acetylene-reduction assay system in short-term experiments. Actively growing suspensions of organisms from a continuous culture were incubated up to 2 hr at various $pO_2$ values and under 0.04 atm. acetylene as described in Methods. The optimum $pO_2$ for acetylene reduction was 0.05 atm. (Fig. 1); activity at normal atmospheric $pO_2$ was very low. The slight activity in the anaerobic treatment was probably due to oxygen dissolved in the culture fluid.

![Fig. 1. Effect of $pO_2$ on acetylene reduction by Mycobacterium flavum 301. Samples from a continuous culture were incubated at 30° with shaking under a range of partial pressures of oxygen and with 0.04 atm. acetylene.](image)

Acetylene reduction by cell-free extracts. Cell-free extracts of Mycobacterium flavum 301, prepared and tested as described in Methods, catalysed the reduction of acetylene to ethylene; the reaction continued up to 45 min. at 30°. There was practically no activity in the cell debris remaining after centrifugation; cell debris resuspended in 0.025 M-tris HCl buffer (pH 7.2) and added to the extract inhibited acetylene reduction. Typical figures for ethylene production after 30 min. were: extract alone, 3.97 n-mole/mg. protein/min.; cell debris alone, 0.02 n-mole/mg. protein/min.; extract and cell debris together 0.66 n-mole/mg. protein/min. Sodium dithionite and an ATP-generating system were required and MgCl$_2$ had a pronounced stimulatory effect (Table 5). Presumably endogenous Mg$^{2+}$ in the extract was insufficient to support full activity. Table 5 includes evidence that the extract was highly sensitive to air: activity fell by over 90% after 15 min. exposure to air.

Pyruvate, and to a lesser extent $\alpha$-ketobutyrate, supply both electrons and energy-rich phosphate for reductions catalysed by nitrogenase in cell-free preparations from
Clostridium pasteurianum (Carnahan, Mortenson, Mower & Castle, 1960) and Bacillus polymyxa (Grau & Wilson, 1961, 1963). The following substrates, with the co-factors indicated, did not replace Na$_2$S$_2$O$_4$ as electron donors for acetylene reduction by Mycobacterium flavum 301 extracts: the ATP-generating system: pyruvate (+ thiamine pyrophosphate, coenzyme A), α-ketobutyrate (+ thiamine pyrophosphate, coenzyme A, NAD), α-ketoglutarate (+ coenzyme A, NAD), succinate (+ coenzyme A, NAD), glucose (+ NAD) and glucose-6-phosphate (+ NAD). Cofactors were added in the following quantities: thiamine pyrophosphate, 100 μg.; coenzyme A, 0.05 μmole; NAD, 1 μmole.

Reduction of nitrogen, cyanide and methyl isocyanide by cell-free extracts. Cell-free extracts catalysed the reduction of nitrogen to ammonia (Table 6). Nitrogenase systems from other organisms catalyse reduction of a variety of substrates besides acetylene and nitrogen: cyanide (Hardy & Knight, 1967) and methyl isocyanide (Kelly, Postgate & Richards, 1967) were chosen as representative alternative substrates. Both were reduced (Table 6). Potassium cyanide yielded methane and traces of acetylene, but no detectable ethane or ethylene. Methyl isocyanide was reduced to methane + traces of

Table 5. Requirements for acetylene reduction by cell-free extracts of Mycobacterium flavum 301

For preparation of cell-free extracts and test conditions, see text.

<table>
<thead>
<tr>
<th></th>
<th>Ethylene produced (%) activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>100</td>
</tr>
<tr>
<td>Complete system - Na$_2$S$_2$O$_4$</td>
<td>5</td>
</tr>
<tr>
<td>Complete system - ATP-generating system</td>
<td>5</td>
</tr>
<tr>
<td>Complete system - MgCl$_2$</td>
<td>30</td>
</tr>
<tr>
<td>Complete system but extract exposed to air 5 min.</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 6. Reduction of nitrogen, cyanide and methyl isocyanide by cell-free extracts of Mycobacterium flavum 301

For experimental details see text; . signifies product not tested for.

<table>
<thead>
<tr>
<th>Substrate (conc.)</th>
<th>NH$_3$</th>
<th>CH$_4$</th>
<th>C$_2$H$_4$</th>
<th>C$_2$H$_6$</th>
<th>C$_3$H$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen (1 atm.)</td>
<td>3.44</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>Acetylene (0.025 atm.)</td>
<td>.</td>
<td>0</td>
<td>5.20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>KCN (2 mM)</td>
<td>.</td>
<td>3.73</td>
<td>trace</td>
<td>0</td>
<td>trace</td>
</tr>
<tr>
<td>Methyl isocyanide (10 mM)</td>
<td>.</td>
<td>5.52</td>
<td>trace</td>
<td>trace</td>
<td>trace</td>
</tr>
</tbody>
</table>

all three C$_2$ compounds. Work by Dr M. Kelly in this laboratory suggests that the acetylene observed was a product of non-enzymic reductive breakdown of the substrate. Ethylene and ethane, however, were formed enzymically; they were produced in equivalent amounts up to 45 min., but in double-scale incubation for 90 min. production of ethane was 50% greater than that of ethylene. Kelly et al. (1967) and Kelly (1968a) reported that the ratios of C$_2$ products, and the proportions of C$_1$ to C$_2$ products, formed from methyl isocyanide by nitrogenase preparations from Azotobacter chroococcum depend on test conditions.

Hydrogenase activity in Mycobacterium flavum 301. All intact nitrogen-fixing systems have hydrogenases (Wilson, 1958). Whole organisms of M. flavum 301 were
tested and conformed to this pattern: they reduced benzylviologen, methylene blue or
tetrazolium after 20 to 30 min. under H₂ but not under argon. Benzylviologen, methy-
lene blue and tetrazolium were reduced by cell-free extracts after 5 to 10 min. under H₂.

Cell-free nitrogen-fixing preparations from a variety of organisms show ATP-
dependent H₂ evolution from dithionite (Burns, 1965). Cell-free extracts from Myco-
bacterium flavum 301 also evolved H₂ (6-89 n-mole H₂/mg. protein/min.) from dithio-
nite when supplied with an ATP-generating system, but H₂ evolution was not ob-
erved in the absence of ATP. Reductant-dependent ATP-ase activity was measured
and, by comparison with a control vessel lacking reductant, the ratio mole ATP
hydrolysed to mole H₂ evolved (ATP/2e⁻) was 3·5.

Pyruvate (250 μmole + coenzyme A, 0·125 μmole; thiamine pyrophosphate,
0·25 μg.; ADP, 6·25 μmole; and Na phosphate, 7·5 μmole; V₀ = 2·5 ml.) did not
support H₂ evolution by cell-free extracts when added in place of sodium dithionite.

Solubility of cell-free extracts. Crude nitrogenase preparations from Azotobacter
vinelandii (Bulen et al. 1964) and A. chroococcum (Kelly, 1966) are particulate: they
sediment after about 4 hr at 200,000g. This property distinguishes them from the
wholly soluble preparations obtained from Clostridium pasteurianum (Carnahan et a.
1960). Ultracentrifugation of cell-free extracts of Mycobacterium flavum 301 for
3·5 hr at 145,000g yielded a pellet which contained all the acetylene-reducing activity.
The supernatant fluid had virtually no activity, nor did it stimulate activity when added
to the resuspended pellet (Table 7).

Table 7. Acetylene reduction by ultracentrifuged extracts of
Mycobacterium flavum 301

<table>
<thead>
<tr>
<th></th>
<th>Ethylene produced (n-mole/mg. protein/min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>0·52</td>
</tr>
<tr>
<td>Pellet from ultracentrifugation</td>
<td>0·54</td>
</tr>
<tr>
<td>Supernatant fluid from ultracentrifugation</td>
<td>0·01</td>
</tr>
<tr>
<td>Supernatant fluid + pellet</td>
<td>0·24</td>
</tr>
</tbody>
</table>

Acetylene reduction without an ATP-generating system. An ATP-generating system
is conventionally used in nitrogenase tests because the system requires ATP but is
inhibited by high ATP concentrations. Moustafa & Mortenson (1967) showed that the
reduction of acetylene by cell-free extracts of Clostridium pasteurianum was supported
directly by ATP without an ATP-generating system. ATP alone also supported
acetylene reduction by extracts of Mycobacterium flavum 301 when the ATP-generating
system was omitted (Table 8). The reaction continued for 12 to 15 min. and re-
quired ATP, Na₂S₂O₄ and MgCl₂; ATP could not be replaced by ADP or AMP. The
optimal concentration of ATP was 4 to 8 mM (Fig. 2) and the inhibitory effect of high
ATP concentrations found in other systems was confirmed. Table 8 includes data
showing that, with optimal ATP, both ADP and AMP were strongly inhibitory; as
mentioned under Methods, the ADP used contained some AMP as impurity. These
results parallel those obtained with the clostridial system.
Fig. 2. Effect of ATP concentration on acetylene reduction by extracts of *Mycobacterium flavum* 301. ATP and Mg$^{2+}$ (as MgCl$_2$) in molar ratio 2:1; for other conditions see text.

**Table 8. Acetylene reduction by cell-free extracts of *Mycobacterium flavum* 301 without an ATP-generating system**

<table>
<thead>
<tr>
<th>Adenosine phosphates (conc.)</th>
<th>Ethylene produced (n-mole/mg protein/min.)</th>
<th>Adenosine phosphates (conc.)</th>
<th>Ethylene produced (n-mole/mg protein/min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>&lt;0.005</td>
<td>ATP (4 mM) + ADP (4 mM)</td>
<td>0.005</td>
</tr>
<tr>
<td>ATP (4 mM)</td>
<td>0.29</td>
<td>ATP (4 mM) + ADP (1 mM)</td>
<td>0.025</td>
</tr>
<tr>
<td>ATP (4 mM) - Na$_2$S$_2$O$_4$</td>
<td>&lt;0.005</td>
<td>ATP (4 mM) + AMP (4 mM)</td>
<td>0.005</td>
</tr>
<tr>
<td>ATP (4 mM) - MgCl$_2$</td>
<td>&lt;0.005</td>
<td>ATP (6 mM)</td>
<td>0.27</td>
</tr>
<tr>
<td>ADP (4 mM)</td>
<td>0</td>
<td>ATP (8 mM)</td>
<td>0.27</td>
</tr>
<tr>
<td>AMP (4 mM)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**DISCUSSION**

*Oxygen-sensitivity of nitrogen-fixing Mycobacterium flavum* 301. Different optimum pO$_2$ values for growth and nitrogen fixation as between batch or continuous cultures, and for acetylene reduction by whole organisms in short-term experiments, merely reflect differences in availability of oxygen to the population being studied, which is determined by many factors, including the geometry of the experimental vessels, the shaking or stirring rates and population densities at any given time. Nevertheless the results reported here clearly indicate that nitrogen-fixing cultures of *M. flavum* 301 tend to be microaerophilic. This conclusion agrees with evidence of numerous other workers, cited by Dalton & Postgate (1969), that oxygen inhibits nitrogen fixation in aerobic organisms. The facultative aerobes, *Bacillus polymyxa* (Hino & Wilson, 1958; Grau & Wilson, 1962) and *Klebsiella pneumoniae* (Pengra & Wilson, 1958), only fix nitrogen anaerobically. *M. flavum* 301 appears to be intermediate in oxygen sensitivity between such organisms and better adapted aerobes such as Azotobacter. Kalininskaya
(1967a) obtained evidence for an inhibitory effect of oxygen on nitrogen-fixing symbiotic microbial associations similar to those from which *M. flavum* 301 was isolated. Nitrogen fixation was stimulated with the development of the symbiotic association, which Kalininskaya (1967c) attributed, at least in part, to protection of the nitrogen fixers from oxygen by the symbionts.

Dalton & Postgate (1969) showed that the sensitivity of Azotobacter species to oxygen inhibition depended on nutritional status. They attributed this phenomenon to the need to protect nitrogenase from damage or interference by oxygen, and postulated two mechanisms of protection: a 'respiratory protection', whereby respiration prevented access of oxygen to the nitrogen-fixing site, and a 'conformational protection'. The latter was reflected by the relative stability to air of the particulate nitrogen-fixing systems extracted from azotobacters (Bulen, Burns & Le Comte, 1964; Kelly, 1966), compared with the soluble, oxygen-sensitive enzyme fractions obtained on fractionating such particles (Bulen & Le Comte, 1966; Kelly, Klucas & Burris, 1967; Kelly, 1968b). They suggested that the oxygen-insensitive particle represented a gross manifestation of the 'conformational protection' mechanism against oxygen in Azotobacter. However, nitrogenase from *Mycobacterium flavum* 301 provides an example of a system which is oxygen-sensitive in the particulate form, which suggests an inefficient or totally absent system of 'conformational protection' in this organism. The microaerophilic character of nitrogen-fixing *M. flavum* 301 may reflect a greater dependence on 'respiratory protection'.

**Enzymology of nitrogenase from Mycobacterium flavum 301.** The cell-free nitrogen-fixing system from *M. flavum* 301 is markedly similar to those from other nitrogen-fixing organisms. It requires: (i) ATP; ADP, AMP or high concentrations of ATP are inhibitory; (ii) a source of low potential reducing power (Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>); (iii) Mg<sup>2+</sup> ions; (iv) anaerobic conditions during preparation and assay. Analogous substrates such as cyanide, isocyanide or acetylene are reduced; ATP-dependent hydrogen evolution and reductant-dependent ATP-ase activity occur, and conventional hydrogenase activity is present.

The ATP/2e<sup>−</sup> ratio of 3.5 observed with *Mycobacterium flavum* 301 extracts is lower than the values of 4 to 5 generally obtained with Azotobacter and *Clostridium pasteurianum* (Bulen & Le Comte, 1966; Mortenson, 1966; Silver, 1967; Winter & Burris, 1968). The system in *M. flavum* 301 may be more efficient, but not only is the validity of the assumptions made in this kind of calculation questionable (Hardy & Burns, 1968), but Kelly (1968b) has shown that the ratio varies greatly according to the experimental conditions and the substrate being reduced. Hence this study only shows that the behaviour of nitrogenase from *M. flavum* 301 with respect to ATP is quantitatively of the same order as the behaviour of extracts of other organisms.

The particulate character of the system from *Mycobacterium flavum* 301 and its failure to utilize pyruvate, recall the system of Azotobacter (Bulen *et al.* 1964; Kelly, 1966) rather than the soluble pyruvate-utilizing system of *Clostridium pasteurianum* (Carnahan *et al.* 1960).
REFERENCES

(Page numbers for Russian references refer to Russian originals; most of those cited are available in English translation)


Nitrogen fixation by Mycobacterium flavum


