

Influence of Partial Pressures of Acetylene and Nitrogen upon Nitrogenase Activity of Species of *Beijerinckia*

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Abstract

Acetylene reduction and nitrogen fixation by strains of *Beijerinckia indica* and *B. lacticogenes* increased with increased partial pressures of acetylene and nitrogen up to 80 kPa. The optical emission spectrophotometric method was used for the determination of ^{14}N : ^{15}N ratios. The molar ratios of acetylene to nitrogen varied greatly from the theoretical value.

Introduction

According to Hardy *et al.* (1973) the use of an acetylene pressure of 10 kPa in assays should produce saturation of nitrogenase in whole cells of nitrogen-fixing microorganisms comparable to a pN_2 of 80 kPa. Most acetylene-reduction assays of nitrogenase activity have used a partial pressure of acetylene between 5 and 20 kPa. However, Spiff and Odu (1973) reported that acetylene reduction by *Beijerinckia indica* (*Azotobacter indicum* ATCC 9037) increased up to a partial pressure of acetylene in the gas phase of 75 kPa. Clearly, the gas phase composition with respect to partial pressures of nitrogen, acetylene, and also oxygen (Drozd and Postgate 1970; Bergersen 1970; Spiff and Odu 1973) can have a profound influence on the rate of acetylene reduction or nitrogen fixation. This paper examines the effects of various partial pressures of acetylene and nitrogen on acetylene reduction and nitrogen fixation by two strains of species of *Beijerinckia*.

Materials and Methods

Materials

Pure gases (oxygen, argon, nitrogen and ethylene) were obtained from Messer Griesheim GMBH, Düsseldorf, and $^{15}\text{N}_2$ (95% enrichment) was purchased from the British Oxygen Co. Ltd, London. Industrial acetylene was purified by washing with concentrated sulphuric acid then water.

Organisms and Growth Conditions

The organisms used in this study were *B. indica* ATCC 9540, and *B. lacticogenes* NCIB 8846. The medium used for the growth of the bacteria had the following composition (in g/l): K_2HPO_4 , 0.03; KH_2PO_4 , 0.07; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.0005; glucose, 20.0, distilled water at pH 6.1, and was dispensed in 50-ml amounts in 250-ml Erlenmeyer flasks. Glucose was autoclaved separately and added to the medium at the time of inoculation. After inoculation the flasks were incubated as static cultures at 30°C. When the cultures had reached the late exponential-stationary phase of growth, the influence of various partial pressures of acetylene and nitrogen upon acetylene reduction and nitrogen fixation was tested.

Acetylene Reduction and Nitrogen Fixation Assay

The assays for acetylene reduction and nitrogen fixation were performed simultaneously with portions from the same batch of cells. The 12-ml serum vials were sealed with serum caps, attached to a high vacuum line via hypodermic needles and evacuated to a pressure of 10 kPa. The gas phase of half of the vials was then made up with 10 kPa oxygen:5–80 kPa acetylene:argon to 100 kPa by adding pure gases with gas-tight syringes. Allowance was made for the injection of 2 ml culture into each of the vials which were then incubated for 1 h in a reciprocating shaker–water bath at 30°C (220 strokes/min). The gas phase was then sampled and the amount of acetylene and ethylene determined by gas chromatography, and the dry weight of cells (usually between 0.5 and 1.0 mg) determined. The production of ethylene was found to be linear over the 1-h incubation period. This was checked by assaying the gas phase in three replicate vials containing the bacterial suspensions at 10-min intervals for 1 h. Three new replicate vials were sampled at each 10-min interval.

The gas phase of the remaining vials was made up with 20 kPa oxygen:5–80 kPa nitrogen:argon to 100 kPa as above. The nitrogen contained from 10 to 44.5 atom % excess ^{15}N depending upon the experiment. After injection of the culture, the vials were incubated in the same way as for the acetylene-reduction assay. After 1 h the gas phase was sampled into evacuated glass tubes for later determination of ^{15}N content by mass spectrometry. The bacterial cells were recovered by centrifugation, and resuspended in 2 ml distilled water. Samples of the bacterial cell suspensions and the supernatants were hydrolysed with 6 M HCl for 48 h at 105°C and nitrogen determined by the ninhydrin method (Spies 1957). This method has been found to correlate well with total nitrogen determination by the Kjeldahl procedure (G. Proksch, personal communication). To enable $^{14}\text{N}:^{15}\text{N}$ ratios to be determined by the optical emission spectrometry technique (Fiedler and Proksch 1972a), the bacterial suspensions were diluted with distilled water to provide the correct amount of nitrogen. Samples (2.9 μl) of the suspensions were taken up into quartz glass capillaries and the electrodeless emission tubes (9 cm by 4 mm o.d.) for optical determination of $^{14}\text{N}:^{15}\text{N}$ ratios were prepared after the methods of Proksch (1972) and Ferraris and Proksch (1972). Following combustion at 800°C in a muffle furnace to bring about the Dumas conversion, the emission tubes were allowed to equilibrate for 12 h before being analysed with a laboratory-modified quartz spectrograph with a mechanical scanning device and photomultiplier. The emitted light was scanned from 297.5 to 298.5 nm to include the bandheads $^{14}\text{N } ^{14}\text{N}$ (297.7 nm) and $^{14}\text{N } ^{15}\text{N}$ (298.3 nm). ^{15}N abundance in the gas phase was determined by crushing the sealed glass sample tubes in the multiple gas sample holder (Fiedler and Proksch 1972b) and analysing the gas using an Hitachi model RMU-6D mass spectrometer. Nitrogen fixation was calculated using ^{15}N enrichment in the assay vial headspace atmosphere, ^{15}N enrichment of bacteria nitrogen (determined by the optical emission technique), and the total nitrogen values for the bacterial suspensions.

Gas chromatographic determination of acetylene and ethylene was carried out with a Perkin Elmer F11 gas chromatograph, having a single steel column (2 m by 5 mm o.d.) packed with Porapak N (80–100 mesh) and a flame-ionization detector. The carrier gas was nitrogen and the column oven temperature was 100°C.

Five replicate vials per treatment were used. The results obtained for the optical emission determination of $^{14}\text{N}:^{15}\text{N}$ ratios were from duplicate electrodeless emission tubes prepared from the bacterial suspensions from each vial.

Results and Discussion

The results of increasing partial pressures of acetylene and nitrogen upon ethylene production and nitrogen fixation respectively by *B. indica* are given for a typical experiment (Fig. 1a). As either acetylene or nitrogen was increased from a partial pressure of 5 to 80 kPa, both acetylene reduction and nitrogen fixation increased. These results are similar to those of Spiff and Odu (1973). The critical partial pressure of acetylene (apparent K_m 6.8 kPa) is greater than the level commonly used in acetylene-reduction studies. Growth of this bacterium was accompanied by very copious production of slime and may have affected the solubility of acetylene. The unusual requirement for a very high partial pressure of nitrogen to saturate the nitrogenase in this bacterium (K_m 5.9 kPa) may also have been due to the

effect of a large amount of slime on diffusion. However, the nitrogen requirement would probably have been even more exaggerated in view of the relative solubilities of nitrogen and acetylene.

The results obtained with *B. lacticogenes* showed a similar trend in that increasing partial pressures of acetylene and nitrogen increased nitrogenase activity significantly (Fig. 1*b*). Results obtained for acetylene reduction by this bacterium showed an unbroken trend of increase in ethylene production with increase in acetylene partial pressure up to 80 kPa acetylene (K_m 7.0 kPa). Growth of this strain was accompanied by slime production, but it was not as copious as with *B. indica*. Data for nitrogen fixation indicate a trend towards increased nitrogen fixation with increased partial pressure of nitrogen (K_m 11.8 kPa).

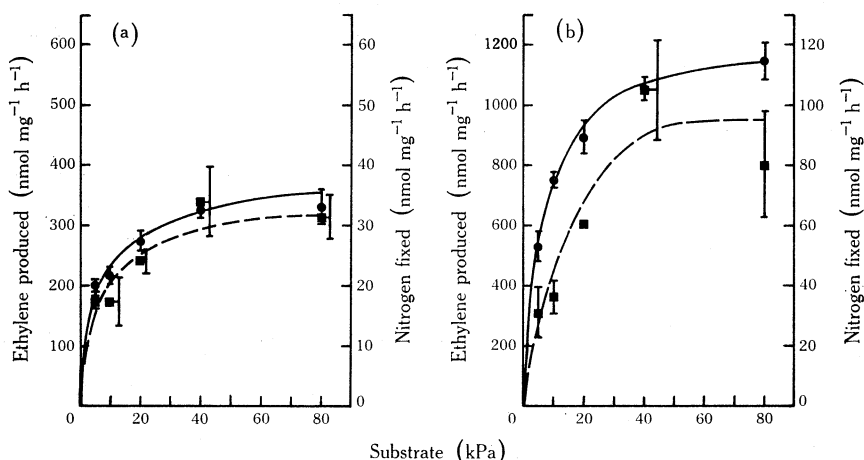


Fig. 1. Acetylene reduction (●) and nitrogen fixation (■) by cultures of (a) *Beijerinckia indica* and (b) *B. lacticogenes* in the presence of increasing amounts of acetylene and nitrogen. Standard errors are shown by vertical bars.

With both strains, the nitrogen fixation data show much more variability than the acetylene-reduction results. The larger errors with the nitrogen data could have been caused by gas sampling problems or by the large multiplication factor used in calculating ¹⁵N enrichment in small subsamples (2.9 µl) of the bacterial suspensions.

Molar ratios of acetylene to nitrogen calculated from the data varied widely. The ranges obtained were 1.9–12.6 for *B. indica* and 10–20.7 for *B. lacticogenes*. Only in the case of some results obtained with *B. indica* did the molar ratios approach the theoretical value of 3 based on electron transfer. Variations in molar ratios for a number of nitrogen-fixing systems have been recorded by Hardy *et al.* (1973).

When the amounts of nitrogen in the supernatants and in the bacterial cells were compared, a very large proportion was found in the supernatant. The percentage of extracellular nitrogen for duplicate experiments was 67.0 and 69.2% for *B. lacticogenes* and 66.6 and 71.4% for *B. indica*.

Although an attempt was made to keep the partial pressure of oxygen constant at 20 kPa, this amount was known to be present only at the start of the incubation period and would have been depleted to some unknown extent during the 1-h assay period. However, it did not affect the rate of acetylene reduction of the two organisms used, as this was found to be linear over the 1-h period. Slime production by the

bacteria may have played a significant role by affecting the availability of acetylene and nitrogen as well as by protecting the nitrogenase from oxygen, as suggested by Hill (1971) in the case of *Derxia gummosa*.

The growth stage of the bacteria may have been a relevant factor affecting molar ratios as the cultures used were in the late logarithmic phase and large amounts of extracellular nitrogen were present. The use of shallow cultures incubated under static conditions was dictated by available facilities, but could well have had an influence upon the molar ratios obtained when small subsamples of the cultures were incubated with shaking during acetylene-reduction and nitrogen-fixation assays.

In view of the major finding in the present study that the nitrogenase of two species of *Beijerinckia* were not saturated at partial pressures of acetylene and nitrogen commonly used in nitrogen-fixation studies, great care needs to be taken in the selection of gas phase composition for both acetylene-reduction tests and nitrogen-fixation studies to ensure maximum nitrogenase activity.

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