Effects of Ammonium Ions, Oxygen, Carbon Monoxide, and Acetylene on Anaerobic and Aerobic Hydrogen Formation by \textit{Anabaena cylindrica} B629

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An investigation was made of various factors, both experimental and physiological, which influenced the formation of hydrogen gas by the heterocystous cyanobacterium \textit{Anabaena cylindrica} B629 when incubated in both argon and air. \textit{A. cylindrica} B629 produces hydrogen in air in the presence of carbon monoxide, acetylene, or both, with a short lag period. The rate of production in air at optimal concentrations of these compounds was found to be comparable with that in an argon atmosphere. Whereas under argon, ammonium ions (0.5 to 6 mM) were found to inhibit hydrogen formation in a manner which was dependent on light intensity and not relieved by oxygen (1 to 20% of gas phase), in air–carbon monoxide–acetylene, these ions (up to at least 0.5 mM) slightly stimulated hydrogen production for at least 24 h. Conclusions are drawn about short-term aerobic and anaerobic hydrogen formation by \textit{A. cylindrica} B629 and the effects of ammonium ions, oxygen, carbon monoxide, and acetylene on these processes.

Hydrogen metabolism in nitrogen-fixing cyanobacteria is currently being investigated for two main reasons. First, biological hydrogen gas formation in general, and from this source in particular, is considered to be a potential source of fuel (4, 20, 26). Second, the efficiency of microbial nitrogen fixation is profoundly influenced by an organism’s ability to metabolize hydrogen (8, 21).

Heterocystous cyanobacteria were initially shown to evolve hydrogen gas in an anaerobic atmosphere in the absence of dinitrogen (1, 24), this process being mediated by nitrogenase enzyme (3, 6, 13). These organisms are capable of nitrogen fixation in air because the heterocyst cells protect the oxygen-labile nitrogenase from oxygen (10), and this fact has enabled manipulation of cultures with carbon monoxide and acetylene such that the organisms release hydrogen in air (6).

In the present study we have investigated four aspects of the hydrogen metabolism by \textit{Anabaena cylindrica} B629. First, we have made a comparative study of the rates of hydrogen formation in argon and air. This aspect is of obvious interest in terms of possible eventual application of the process in biological solar energy conversion. Rates of aerobic hydrogen formation reported to date (6, 16) have been significantly less than those obtained in argon, possibly due to the presence of non-optimal CO and C$_2$H$_2$ concentrations (2 and 10% of the gas phase, respectively). Second, we have clarified the effect of ammonium ions on hydrogen formation in argon, over a range of concentrations and at different light intensities. Although long-term effects of ammonium ions on hydrogen production in the present study are fairly well documented (12, 31), short-term effects over a few hours of incubation have not received attention. Third, the effect of exposing cultures to ammonium ions and oxygen simultaneously has been investigated. The possibility of a synergistic effect of ammonium ions and oxygen, wherein either alleviates inhibition by the other, as occurs in many other nitrogen-fixing microorganisms (27), has not previously been examined with \textit{A. cylindrica}. Fourth, as part of the comparative study of anaerobic and aerobic hydrogen formation, we have studied the effect of ammonium ions in air–CO–C$_2$H$_2$.

**MATERIALS AND METHODS**

Algae and their growth. \textit{A. cylindrica} B629, bacteria-free, was obtained from the culture collection of algae, University of Texas at Austin, and grown as described previously (6, 16). Cultures were harvested at an initial density in the range of 50 to 140 Klett units. Filaments were harvested by settling and resuspended to give the desired final concentration. Concentrations were also determined in terms of dry...
weights by heating duplicate 10-ml samples for 16 h at 85°C and correcting for the weight of mineral salts in the medium.

Incubations. Algae (10 ml) at appropriate concentrations in 200-250 Klett units were pipetted into Erlenmeyer flasks (36 ml capacity) fitted with rubber subaseals (no. 41, W. Freeman and Co., Yorkshire, England). Gas phases were set up as described below, and the algae were incubated with shaking, at 25°C under a light intensity of 4,000 lux.

Gas phase and gas analysis. When the outer rubber on the subaseals was downturned in the usual way, problems were encountered due to gas leakage through the subaseal, particularly through the insertion points of larger needles when the internal flask pressure differed from atmospheric pressure. The simple precaution of leaving the subaseal's outer rubber upturned and containing 1 to 2 ml of water in no way interfered with sampling and proved very effective in eliminating this variable source of error. Any leakage of gas from, or into, a flask could be clearly seen, and the faulty subaseal was discarded.

It was found that the common procedure of vacuum degassing in the absence of physical agitation of the solution was inadequate for the removal of dissolved gases because of the slowness of phase transfer of residual gases such as hydrogen, ethylene, or acetylene dissolved in the solutions. Only when vacuum degassing was coupled with vigorous shaking did sufficiently rapid transfer of gases occur (Fig. 1). Phase transfer of acetylene and ethylene was even slower than that of hydrogen, but again agitation was effective in ensuring rapid phase transfer of these gases. It has previously been shown (9) that neglecting phase transfer problems can be a serious source of error in relation to acetylene reduction measurements.

The gas atmospheres in the flasks were made by inserting through the subaseals 24 gauge needles that were connected to a vacuum pump, manometer, and gas cylinders via a manifold system. The manifold was attached to a shaker on to which 20 flasks could be clamped. A shaking rate of 120 oscillations per min for 15 min with simultaneous degassing by the pump was sufficient to ensure removal of residual dissolved gases; at 5-min intervals during this period, the flasks were quickly refilled to atmospheric pressure with the appropriate gas. Although this procedure was used in all experiments reported herein, because it was both simple and rapid, we found that vigorous bubbling of argon through solutions was also a satisfactory method of facilitating gas phase exchange.

Gases other than the predominant one, such as CO, C\textsubscript{2}H\textsubscript{2}, CO\textsubscript{2}, N\textsubscript{2}, and O\textsubscript{2}, were injected separately with syringes prefilled with the appropriate gas. In preliminary experiments, such injection, by causing an overpressure within the flasks, resulted in a partial loss of the gas sample from syringes during their transfer from the incubation flask to a rubber stopper in which they could be stored. This was demonstrated conversely by measuring the leakage of atmospheric oxygen and nitrogen into a syringe due to a pressure drop within the syringe caused by repeated sampling from a flask initially filled with argon to atmospheric pressure; these results were in good agreement with theoretical expectations assuming no free mixing of the gas in the syringe with air but complete equilibration of the syringe to atmospheric pressure (results not shown). Hence, to ensure that the injection into flasks of these minor gases restored atmospheric pressure, we withdrew a calculated volume of gas from the flasks before their injection. It is inadequate to withdraw a volume of gas equal to that to be injected subsequently because the volume withdrawn will be at a pressure less than atmospheric. The relationship between the volume to be withdrawn (y) and that to be subsequently injected (x) to give atmospheric pressure is y = a + bx = 0, where V is the gas volume in the flask; this equation is simply derived from Boyle's Law. If this relationship were to be used for highly soluble gases such as CO\textsubscript{2}, or for flasks with a high liquid-to-gas volume ratio, an additional term containing the solubility coefficient would have to be included. Although gas mixing devices can be used (30) which allow restoration of atmospheric pressure manometrically, with highly soluble gases such as CO\textsubscript{2} and gases required at low concentrations, separate injection is required.

A related problem arises from overpressure which occurs due to production of gas by microorganisms during an experiment. This can be made insignificant by selecting an appropriately low concentration of algal suspension as in the experiments described herein. Alternatively, the pressure can be relieved by inserting an expansion syringe into the flasks, either continuously in longer-term experiments or just before gas sampling; in the latter case, because slow diffusion of hydrogen through the needle resulted in errors, the procedure adopted was to insert the syringe through the subaseal, allow it to expand, measure the volume

![Fig. 1. Equilibration of dissolved H\textsubscript{2} into the gas phase from 10 ml of Allen and Arnon medium in 37-
ml Erlenmeyer flasks fitted with rubber stoppers (subaseals). Flasks were either shaken at 120 oscillations per minute (○) or left stationary (●). The solutions containing H\textsubscript{2} were prepared by preincubating flasks containing pure H\textsubscript{2}, the H\textsubscript{2} in the gas phase then being removed by vacuum degassing without shaking and quickly replaced with argon. The total H\textsubscript{2} in 10 ml of H\textsubscript{2}-saturated medium (---) was calculated from the solubility coefficient of H\textsubscript{2} at 25°C (30).](http://aem.asm.org/)

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of expanded gas, leave the expansion syringe positioned while a sample was taken for analysis, and then reinject the expanded volume back into the flask. We found the most responsive syringes to be of glass. It is of interest that an analogous procedure in which photosynthetic bacteria are incubated within syringes has recently been reported (3).

At appropriate times during the experiments 1-ml gas samples were withdrawn with disposable plastic syringes (28-gauge needles). For measurements of acetylene reduction, unless otherwise stated, an initial acetylene concentration of 10% in the gas phase was used. Preliminary experiments showed this concentration to be optimal (Fig. 2). Measurements such as these are important because species vary in the nature of their saturation curves for acetylene (19). Ethylene was determined as described previously (6). Hydrogen, oxygen, and nitrogen were determined with a Pye Katharometer 104 series gas chromatograph (6). Before gas chromatographic analysis, syringes were inserted into rubber stoppers for storage. That no loss of hydrogen occurred during storage was checked by storing known amounts of hydrogen for different times in new and well-used rubber stoppers. Only a very slow leakage of hydrogen occurred in the former case but a variable, and often pronounced, loss occurred with stoppers previously perforated with needles. The need for replacing stoppers regularly was indicated.

Finally, because several gas samples were often taken from a given flask in an experiment it was important to correct measurements made at a given time for gas lost in previous samplings.

RESULTS

Rates of aerobic hydrogen formation as a function of CO or C$_2$H$_2$ concentrations or both. The rates of hydrogen formation in air-3% CO$_2$ were measured in the presence of various concentrations of CO and C$_2$H$_2$ alone, and as CO was varied in the presence of C$_2$H$_2$ (10%) or as C$_2$H$_2$ was varied in the presence of CO (0.2%). CO is known to inhibit all but the H$_2$-evolving activities of nitrogenase, including nitrogen fixation (32), and C$_2$H$_2$ is known to inhibit H$_2$ recycling (2, 3) and may also enhance nitrogenase activity. The concentrations of CO and C$_2$H$_2$ were each varied in air in the absence or presence of a fixed excess of the other. The rates obtained from 5 to 24 h of incubation are shown in Fig. 3; although rates were low (<30 nmol/mg per h) when CO or C$_2$H$_2$ were varied alone (Fig. 3A), rates of up to 200 nmol/mg per h occurred as C$_2$H$_2$ was varied in the presence of 0.2% CO or as CO was varied in the presence of 10% C$_2$H$_2$ (Fig. 3B). Hence, CO and C$_2$H$_2$ together exert a synergistic effect in enhancing aerobic hydrogen formation. The maximal rate in air (Fig. 3) is to be compared with maximal rates of 100 to 200 nmol/h per mg of cyanobacteria commonly obtained in an argon atmosphere. The optimal concentrations of CO and C$_2$H$_2$ are ca. 0.2 and 5%, respectively (Fig. 3B). The time course of this aerobic hydrogen formation showed a lag period of several hours (Fig. 4), after which hydrogen formation was linear for at least 20 h. This lag presumably reflects the time taken for CO and C$_2$H$_2$ to exert their combined metabolic effects. The lower rate of H$_2$ formation at CO concentrations of <0.1% presumably is due to incomplete inhibition by CO of nitrogen fixation; the decreasing rate of H$_2$ evolution as the CO level is elevated from 0.1% probably reflects CO inhibition of a related process such as photosynthesis or respiration.

Acetylene, of course, is normally reduced by nitrogenase enzyme, at substantial rates; however, this reduction was potently inhibited by
FIG. 3. Rates of hydrogen production by A. cylindrica in air–3% CO₂ measured from 5 to 24 h of incubation (A) as C₂H₂ (○) and CO (●) alone are varied and (B) as C₂H₂ is varied in the presence of 0.2% CO (○) and as CO is varied in the presence of 10% C₂H₂ (●). Results are expressed per milligram, dry weight; each flask contained 5.6 mg.

CO (Fig. 5). From the viewpoint of minimizing acetylene reduction, higher CO concentrations are desirable (Fig. 5), but these are not optimal for hydrogen evolution (Fig. 3B). For example, 0.2% CO is near optimal for H₂ evolution, giving a rate of 180 nmol/h per mg of algae (Fig. 3B), but inhibition of C₂H₂ reduction is only about 83% at this CO level (Fig. 5).
Effect of ammonium ions on hydrogen formation in argon. The effect of NH₄Cl (0.5, 3, and 6 mM) and chloramphenicol (20 μg/ml) on hydrogen formation at different light intensities (700, 4,000, and 44,000 lux) was studied (Fig. 6). The rate of hydrogen formation at 4,000 lux was more than twice that at 700 lux and more than three times that at 44,000 lux. In each set of conditions inhibition by ammonium ions did not appear to be markedly concentration dependent in the range studied (0.5 to 6 mM), particularly within the first 2 h. Of particular interest is the fact that inhibition by ammonium ions, when compared to controls in argon, becomes progressively less as the light intensity is increased (Fig. 6A, B, and C). By contrast, the extent of inhibition by the protein synthesis inhibitor chloramphenicol exhibited no such light dependence. That is, at 700 lux (Fig. 6A) chloramphenicol proved less inhibitory than ammonium ions; at 4,000 lux (Fig. 6B) inhibition was comparable; at 44,000 lux (Fig. 6C) chloramphenicol proved more inhibitory than ammonium ions. Of interest was the fact that the addition of both ammonium ions and chloramphenicol in each case resulted in a more pronounced inhibition than that effected by either alone (results not shown). The effect of ammonium ions or chloramphenicol under each of these conditions was more marked upon hydrogen formation than on acetylene reduction (latter results not shown).

Effect of simultaneous exposure to oxygen and ammonium ions in argon. Both O₂ (20%) and NH₄Cl (0.5 mM) alone inhibited H₂ formation by A. cylindrica, and O₂ (20%) failed to alleviate the inhibition by NH₄Cl as measured over 4 h of incubation (Table 1). The possibility that lower O₂ tensions (1 to 10%) might relieve NH₄⁺ inhibition, measured over a longer incubation period of 23 h, was investigated. Low O₂ tensions of 1 and 2% alone did not inhibit H₂ evolution as measured after either 2 or 23 h, although higher O₂ tensions of 5 and 10% inhibited H₂ formation by more than 50% after 23 h of incubation (Fig. 7A to E). Inhibition of H₂ formation by 0.5 mM NH₄Cl was marginal by 2 h but pronounced by 23 h of incubation (Fig. 7F). When 0.5 mM NH₄Cl and O₂ were both present, no relief of NH₄⁺ inhibition was evident, regardless of O₂ tension (1 to 10%) or whether cultures were sampled at 2 or 23 h of incubation (Fig. 7G to J).

Effect of ammonium ions on aerobic hydrogen formation in the presence of CO and C₂H₂. NH₄Cl (at a concentration of at least 0.5 mM) slightly stimulated hydrogen formation under these conditions when measured from 4 to 24 h of incubation (Fig. 8). Rates were measured only from 4 h onward because by this time the lag evident in Fig. 4 was complete. In some experiments the stimulation was as much as 50% over this incubation period. This is in marked contrast with the potent inhibition by 0.5 mM NH₄Cl beneath an argon atmosphere over a similar time period (Fig. 7F). To determine whether this was due simply to some effect of CO and C₂H₂, the effect of 0.5 mM NH₄Cl on H₂ formation beneath an argon atmosphere in the absence and presence of CO (0.2%) and C₂H₂ (5%) was studied; in the presence of 0.5 mM NH₄Cl, hydrogen formation was inhibited to a similar extent in each case (Table 2). Interestingly, hydrogen formation in argon-CO-C₂H₂-CO₂ exhibited no lag period (results not shown) as opposed to the pronounced lag in air-CO-C₂H₂-CO₂ (Fig. 4). Also, CO and C₂H₂ did not stimulate hydrogen formation in argon-CO₂ in the absence of NH₄Cl (Table 2).

DISCUSSION

The present work extends that reported earlier (6), which showed that addition of CO and C₂H₂ allows H₂ formation in an aerobic environment, in determining the optimal concentrations of these compounds and showing that under such optimal conditions, after a lag period, rates can be obtained which are comparable with those in an anaerobic environment of argon (Fig. 3). Although CO is likely to act at multiple sites in the cell, its primary role in these experiments is as an inhibitor of all but the hydrogen-forming activities of nitrogenase (29) and as a partial inhibitor of uptake hydrogenase activity (28). The role of acetylene is less clear. Although in certain nitrogen-fixing microorganisms it has been demonstrated to enhance hydrogen production by inhibiting uptake hydrogenase activity (28) and has been assumed to act as such in
cyanobacteria (2, 3, 6), recent work has shown that acetylene may not inhibit either the hydrogen-evolving (5) or hydrogen-consuming (25) reactions of hydrogenase enzymes. Part of the stimulation by acetylene may be due to a direct effect on the nitrogenase enzyme itself (7, 18).

An understanding of the effect of ammonium ions is essential for microorganisms in which hydrogen formation is essentially nitrogenase mediated. It is well-established that addition of NH$_4^+$ to the medium leads to repression of nitrogenase synthesis in cyanobacteria (10). It has recently been shown that it is not NH$_4^+$ itself but a metabolic derivative such as glutamine which is the repressor, since addition of methionine sulfoximine (29) or 5-hydroxylysine (14), inhibitors of glutamine synthesis, alleviates the inhibitory effect of NH$_4^+$. In addition, NH$_4^+$ ions also cause an immediate inhibition of nitrogenase, which can apparently be explained in terms of a redirection of reductant, adenosine 5'-triphosphate (ATP), or both, away from the nitrogenase by competing biosynthetic pathways involving NH$_4^+$. (22, 23). This conclusion is confirmed by Fig. 6, which shows that the inhibitory effect of NH$_4$Cl, compared with that of the pro-

**Table 1. Effect of NH$_4$Cl on hydrogen formation in the presence and absence of oxygen**

<table>
<thead>
<tr>
<th>Gas atmosphere $^a$</th>
<th>NH$_4$Cl $^b$ (0.5 mM)</th>
<th>H$_2$ produced in 4 h (nmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argon–3% CO$_2$</td>
<td>–</td>
<td>770</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>270</td>
</tr>
<tr>
<td>Argon–3% CO$_2$–20% O$_2$</td>
<td>–</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>30</td>
</tr>
</tbody>
</table>

$^a$ Argon was used to restore atmospheric pressure in each flask.

$^b$ Presence and absence of NH$_4$Cl are denoted by + and –, respectively.

**Fig. 6.** Hydrogen production by algae incubated beneath argon–3% CO$_2$ at 700 lux (A), 4,000 lux (B), and 44,000 lux (C). Cultures were both unsupplemented (○) and supplemented with 0.5 mM (●), 3 mM (□), and 6 mM NH$_4$Cl (■) or with 20 µg of chloramphenicol per ml (△). In each flask the dry weights of cyanobacteria were 4.7 mg (A), 7.3 mg (B), and 6.7 mg (C).
protein synthesis inhibitor chloramphenicol, is greater at low light intensities where reductant and ATP supply would be reduced. It is noteworthy that the inhibitory effect of NH₄Cl on hydrogen formation (Fig. 6) was more pronounced than that on acetylene reduction (result not shown; 29), presumably because hydrogen produced by nitrogenase is recycled to allow recovery of reductant, whereas ethylene is not. This is interesting in that it illustrates that H₂ recycling may be subject to metabolic control. Methionine sulfoximine, added simultaneously with NH₄Cl, only partially alleviates this short-term inhibition (results not shown), presumably because its entry into the cell is relatively slow. These results are not explicable in terms of ammonium ions uncoupling photophosphorylation (10).

In *Rhizobium* species the rapid inhibition of nitrogenase by NH₄⁺ is relieved by O₂, even though each alone is inhibitory (27). This effect is explained in terms of oxygen's role in allowing an increase in the ATP supply to nitrogenase, thereby overcoming its limitation when NH₄⁺ is being rapidly incorporated into amino acids. The experiments reported herein suggest that the interaction between NH₄⁺ and O₂ is different in cyanobacteria as suggested previously in relation to acetylene reduction measurements (22). In an argon atmosphere a range of oxygen tensions failed to alleviate the inhibitory effect of NH₄⁺ (Table 1; Fig. 7). Photosynthetic nitrogen-fixing organisms are clearly different from nonphotosynthetic organisms in this respect, representing the fact that they can use photosynthesis rather than respiration for ATP synthesis. Oxygen might also represent a drain on reductant supply to the heterocysts (17).

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**Fig. 7.** *H₂* production by algae incubated beneath argon-3% CO₂ alone (A) or also in the presence of 1% (B), 2% (C), 5% (D), and 10% O₂ (E) in the gas phase. Corresponding experiments were also done in which the solutions were made 0.5 mM with respect to NH₄Cl and with 1% (G), 2% (H), 5% (I), and 10% (J) O₂ present in the gas phase. *H₂* was measured after either 2 h (part 1) or 23 h (part 2) of incubation.

**Fig. 8.** Rates of *H₂* production by algae incubated beneath an initial atmosphere of air-5% C₂H₂-0.2% CO₂-3% CO₂ as NH₄Cl is varied. Measurements were made between 4 and 24 h of incubation.
TABLE 2. Effect of NH4Cl on hydrogen formation in argon with or without CO and C2H2

<table>
<thead>
<tr>
<th>Gas atmosphere</th>
<th>NH4Cl (0.5 mM)</th>
<th>H2 produced in 3 h (nmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argon–3% CO2</td>
<td>–</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>70</td>
</tr>
<tr>
<td>Argon–3% CO2–0.2% CO–5%</td>
<td>–</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>70</td>
</tr>
</tbody>
</table>

More difficult to explain is the difference between the argon and air results. In the last atmosphere, in the presence of CO and C2H2, NH4+ stimulated H2 formation (Fig. 8). The fact that hydrogen formation is stimulated by 0.5 mM NH4Cl in air–CO–C2H2–CO2 (Fig. 8) but inhibited by 0.5 mM NH4Cl in both argon–CO2 and argon–CO–C2H2–CO2 (Table 2) indicates that the stimulation observed is not simply an effect of CO, C2H2, or both but involves an effect of O2, N2, or both. A complete explanation of this observation can probably only be made when the role of acetylene is better understood. It is, nevertheless, an important observation in terms of attempts being made (15) to maximize H2 production in longer-term experiments in air.

In conclusion, we have evaluated, both under anaerobic and aerobic conditions, the effects and interactions of those substances which directly influence H2 formation under anaerobic conditions. NH4+, O2, CO, and C2H2, each of which alone is in some way inhibitory to metabolic reactions involving H2, in combination allow significant enhancement of rates of H2 formation. In an accompanying paper (15) we report their effects on the duration and long-term rates of H2 formation by this cyanobacterium.

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LITERATURE CITED


