Hydrogen production by algae

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I. The enzymes catalyzing formation or uptake of molecular hydrogen

A variety of microorganisms can evolve H_2 according to the following equation: $2H^+ + 2e \rightleftarrows H_2$. These include strict or facultative anaerobic bacteria, aerobic bacteria, blue-green and green algae. In aerobic bacteria and in blue-green algae H_2 formations are restricted to N_2-fixing species. Strict and facultative anaerobic bacteria as well as green algae *(Chlamydomonas, Scenedesmus, Chlorella)* form the gas only under O_2 exclusion in the cultures. There is no clear-cut demonstration for H_2-formation by mosses, ferns and higher plants. Lists of the H_2-forming organisms are compiled in Mortenson and Chen[1] and Schlegel and Schneider[2].

Since the redox potential of the couple $2H^+/H_2$ is -413 mV at pH 7.0, a low potential reductant is required for H_2-formation to proceed in the cells. The reaction is also enzyme mediated. Cells may contain 3 clearly distinguishable enzymes catalyzing either uptake or evolution of H_2 under physiological conditions (for a more detailed account and the references see Bothe and Eisbrenner[3]).

a) *Reversible, 'classical' hydrogenase*

This soluble enzyme has been characterized best from the anaerobic bacterium *Clostridium pasteurianum.* In the isolated state it catalyzes both uptake and evolution of the gas which are independent of ATP and severely affected by CO and O_2. The enzyme has a mol. wt of about 60,000 and a prosthetic group consisting of three 4 Fe:4 acid labile sulphur centres among which only one is believed to undergo oxidation/reduction during catalysis. Carriers that supply the electrons for H_2-formation by hydrogenase are the iron-sulphur protein ferredoxin or, under iron-deficiency in the culture medium, the flavoprotein flavodoxin. In vitro, ferredoxin and flavodoxin can artificially be substituted by viologen dyes (methyl or benzyl viologen). Continuous H_2-formation requires a continuous supply of reducing equivalents for the reduction of H^+ and ferredoxin. In *Clostridium,* the reducing equivalents are supplied by the electron donors pyruvate or NADH. In the presence of coenzyme A, pyruvate is split to acetylcoenzyme A and CO_2, and the remaining 2 electrons reduce ferredoxin or flavodoxin. This so-called pyruvate phosphoroclastic reaction is catalyzed by the enzyme pyruvate:ferredoxin oxidoreductase. Alternatively, reduced ferredoxin can be generated from NADH in a reaction catalyzed by NADH: ferredoxin oxidoreductase which is allosterically regulated by acetylcoenzyme A in *C. pasteurianum.* Under physiological conditions, the formation of H_2 is the favored reaction in *C. pasteurianum.* Hydrogenase functions by removing excess reducing equivalents generated during fermentation. Since this bacterium cannot degrade carbohydrates completely to CO_2 and H_2O due to the absence of the respiratory chain, it must produce large amounts of H_2 in order to avoid overreduction.

The occurrence of this soluble hydrogenase has been established for saccharolytic *Clostridia* and for facultative anaerobic bacteria (e.g. *Bacillus polymyxa*). A similar enzyme is present in the photosynthetic *Chromatium.* Hydrogenase of the Enterobacteriaceae (e.g. *Escherichia coli*) is part of the membrane-bound formate:hydrogen lyase complex which has not yet been fully characterized. The same complex has recently been demonstrated in the photosynthetic Rhodospirillaceae (e.g. *Rhodospirillum rubrum*). The hydrogenase from green algae is apparently soluble and possibly couples with ferredoxin.

In regard to blue-green algae, a hydrogenase catalyzing H_2-formation under anaerobic conditions in the cells has been demonstrated unequivocally only for the halophytic *Oscillatoria limnetica.* Its occurrence has also been suggested in aerobic, N_2-fixing blue-green algae *(Nostoc muscorum, Anabaena cylindrica);* but this is likely an artifact of cell-free preparations[4]. A soluble, reversible hydrogenase has not been found in aerobic bacteria, including the N_2-fixing species.

b) *Nitrogenase*

The enzyme catalyzes the reduction of N_2 to ammonia, of C_2H_2 to C_2H_4 and of other substrates which have a triple bond in common[5]. In addition, it converts protons and electrons to molecular hydrogen. H_2-formation by nitrogenase is irreversible, insensitive to CO, dependent on a supply of electrons from reduced ferredoxin and requires large amounts of ATP. In vitro, 3-4 molecules of ATP are hydrolyzed for the formation of one molecule of H_2, and in vivo H_2-productions are probably even more energy consuming. The mechanism of H_2-formation by nitrogenase is not understood at present. In the absence of any other substrate, e.g. under argon, all the electrons flowing to isolated nitrogenase reduce H^+ to H_2 despite the low H^+-concentration in such assays normally performed at pH 7-8. Even in the presence of N_2 in the vessels, H_2-evolution is still substantial. The measured stoichiometry between N_2-reduction and H_2-formation is often 1:1, indicating that both reactions are coupled according to the following equation: $8H^+ + 8e^- + N_2 \rightarrow 2NH_3 + H_2$. H_2-production is

always observed with isolated nitrogenases but is often marginal in intact organisms.

c) *Uptake hydrogenase*

Although described already in the early 1940's, the biochemical properties of this enzyme are largely unknown at present. It is an integral protein of membranes and therefore difficult to characterize. It is virtually insensitive to oxygen and has a high affinity for H_2. In nitrogen-fixing cells, it recycles all or most of the H_2 lost by the ATP-dependent formation of H_2 catalyzed by nitrogenase. This explains the low net H_2-formation rates of most aerobic N_2-fixing organisms. The recycling of H_2 has at least 3 beneficial functions for the cells: 1. it provides the organisms with extra ATP. H_2-consumptions proceed by an oxyhydrogen (Knallgas) reaction which is coupled to the respiratory electron transport and to ATP-formation. 2. there is experimental evidence that the oxyhydrogen reaction removes oxygen from the nitrogenase site and thereby protects the enzyme from damage by this gas. 3. H_2 and the uptake hydrogenase can supply electrons for the reduction of N_2 to ammonia by nitrogenase or for the conversion of CO_2 to carbohydrates by the Calvin cycle. The latter reaction has recently been demonstrated in *Rhizobium* and *Derxia gummosa*. H_2-supported nitrogen fixation (C_2H_2-reduction) is particularly pronounced in the heterocysts of blue-green algae where H_2 is an effective electron donor in a strictly light-dependent reaction. Experimentally unverified is a 4th possible function of the uptake hydrogenase. N_2-reduction catalyzed by nitrogenase is affected by high concentrations of H_2. The uptake hydrogenase may, therefore, remove the deleterious H_2 unevitably formed with N_2-reduction by nitrogenase. It is, however, questionable whether the high inhibitory concentrations of H_2 are reached at the nitrogenase site.

Growth under N_2-fixing conditions drastically enhances the activity levels of the uptake hydrogenase in the organisms. The enzyme is, however, not restricted to N_2-fixing cells, since it can be demonstrated in non N_2-fixing blue-green algae and in aerobic, H_2-oxidizing bacteria *(Alcaligenes, Paracoccus, Xanthobacter)*. The electron acceptor for H_2-utilization by the membrane-bound uptake hydrogenase has not been clearly identified in any of the organisms. Inhibitor studies indicate that the electron entry is at or close to the quinone site in respiration and photosynthesis and at a redox level of about 0 volt. This reflects the unidirectional nature of the enzyme; the potential gap between the quinone/hydroquinone and the H^+/H_2 couples prevents the formation of H_2 by this enzyme under physiological conditions. In the isolated state, the enzyme is, of course, able to catalyze H_2-formation provided high concentrations of strong reductants (methyl viologen reduced by excess of $Na_2S_2O_4$) are supplied to the assays. Strains of *Alcaligenes eutrophus* are unique in containing 2 different uptake hydrogenases[2]. In addition to the membrane-bound hydrogenase, these bacteria form a soluble, flavin containing enzyme catalyzing the reduction of NAD^+ by H_2.

II. *Comparison of the capabilities of organisms to produce H_2*

As has already been mentioned, many obligate or facultative anaerobic bacteria ferment organic substrates to H_2. However, none of them is able to degrade organic matter completely to CO_2 and H_2[6]. The highest yield ever measured was 4 mole of H_2 formed from 1 mole of hexose (e.g. glucose). Such findings are in accord with theoretical considerations arguing that 4 mole is the maximal achievable amount[6]. This value decreases to about 2.6 mole of H_2 per mole of glucose when cultures are growing under a H_2-pressure of ≥ 1 at. 4 mole of H_2 contain only 33% of the combustible energy of glucose and 2.6 mole approximately 20%. These figures have to be compared with those for CH_4-formation. 85% of the energy is conserved when CH_4 is the end product of glucose degradation. Energetically it is, therefore, much more efficient to produce CH_4 instead of H_2 when organic matter is to be converted to energy by microorganisms (see Thauer[6]).

The situation may, however, be different with photosynthetic organisms which use solar radiation to build up chemical energy. Photosynthetic bacteria use either inorganic sulphur compounds (Chromatiaceae) or organic substrates (Rhodospirillaceae) as the source of electrons for the photoreduction of CO_2 or, alternatively, photoproduction of H_2. They evolve H_2 in relatively high amounts in a nitrogenase-dependent reaction. Since they do not produce O_2 photosynthetically, H_2 and O_2 must not be separated from each other. Rhodospirillaceae are easily manipulated genetically. However, since their nitrogenase is rather sensitive to exposure to O_2, all photohydrogen production must be performed under strict anaerobic conditions. Photosynthetic bacteria are ubiquitous in nature but appear seldom in blooms and usually show sluggish growth in natural environments. They may be considered when waste material is to be converted to produce H_2. For such a purpose, they have to be grown on a large scale basis under anaerobic conditions and at a defined supply with substrates and light. They do not appear, therefore, to be likely candidates for solar energy conversion programs. Somewhat contrary views have recently been expressed in review[31,32] where photosynthetic bacteria were claimed to currently show the most promise for short-term applied systems.

Green and blue-green algae appear to be more rewarding, at least at first glance. Both groups of organisms are very different in cytological respect;

blue-green algae are of prokaryotic nature and green algae are true eukaryotes like the higher plants. The only property which they share is the capability to perform plant-type photosynthesis. They are able to utilize sunlight and CO_2 for carbohydrate formation at the expense of water as the electron donor. They use two photosystems to generate a strong reductant with a redox potential of about -500 mV (see fig. 1). The chemical nature of this electron carrier X has not been entirely resolved, but it is likely a membrane - bound iron-sulphur protein. This compound X reduces ferredoxin, which is the reductant in several reactions (fig. 1). Ferredoxin donates electrons either to NADPH:ferredoxin oxidoreductase and $NADP^+$ for CO_2-fixation or to nitrite and thiosulphonate reductases in assimilatory nitrate and sulphate reductions. In blue-green algae, a plant-type ferredoxin reduces nitrogenase for the conversion of N_2 to NH_4^+ or H^+ to H_2 [7]. It is conceivable that photoreduced ferredoxin may transfer electrons also to hydrogenase, since many of the reversible hydrogenases couple with ferredoxin (see section Ia). Indeed, H_2-formation by green algae may be dependent on ferredoxin and a classical hydrogenase[8], although the biochemistry of this reaction awaits further elucidation.

The energetic efficiency of solar energy conversion by the photosynthetic electron transport (in the generation of reduced ferredoxin from water) ranges between 8 and 10%, referred to the radiation energy reaching the earth surface[9,10]. The efficiency of the conversion to plant biomass is only 1.0–1.3%. Such low figure is mainly due to energy losses which inevitably occur when CO_2 is reduced to carbohydrates in the Calvin cycle. A great portion of the energy is also used up to supply the plants with water and nourishment. It is the hope to couple the photosynthetic electron transport and reduced ferredoxin to hydrogenase to liberate the energy as H_2 with maximal output. Obviously only artificial systems can proceed with an efficiency of 8–10%, however, they suffer from their inherent instability. Living organisms are stable but have to be manipulated in order to release the captured energy as H_2 with maximal possible yield. For this final goal, blue-green algae offer a better starting position than green algae.

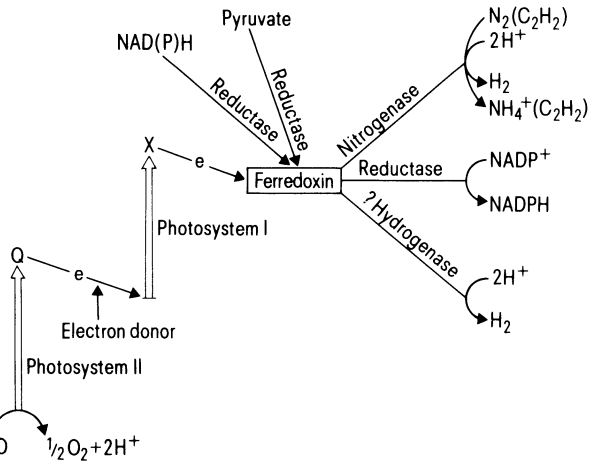

Figure 1. Scheme of the photosynthetic electron transport in thylakoids of green plants and blue green algae.

Green algae produce H_2 only when the cells have been adapted to anaerobic conditions[11–13]. The length of this adaptation process for maximal H_2-formation varies from organism to organism. Adaptation may cause the activation of a constitutive hydrogenase or the synthesis of new enzyme molecules, depending on the algal strain used. In the dark, green algae evolve only minuscule amounts of H_2. The production of the gas is stimulated by organic substrates and is accompanied by a release of CO_2 with a stoichiometry of 2.2:1 between H_2 and CO_2. H_2-formation in the dark is inhibited to uncouplers indicating the involvement of an energy-dependent reverse electron flow in the degradation of carbohydrates to H_2. Light stimulates H_2-evolution of anaerobically adapted green algae. The photoproduction of H_2 is enhanced by uncouplers and is therefore energy-independent. The source of electrons in the cells for the light-dependent H_2-formation is not entirely clear at present. H_2-evolution is not accompanied by a stoichiometric release of O_2. Thus reducing equivalents may be generated partly from endogenous carbon reserves and partly from the photosynthetic water splitting reaction.

Any program for solar energy conversion by green algae is immediately faced with the extreme sensitivity of H_2-formation and hydrogenase towards O_2. H_2-formation capability is entirely lost when the level or O_2 in the assays exceeds 1% of the atmospheric concentration. Since the algae evolve O_2 photosynthetically, H_2 is only formed at low light intensities where hydrogenase synthesis can compete with its destruction by O_2 from photosynthesis. This means that the efficiency of solar energy conversion is very low.

Many blue-green algae (cyanobacteria) need only light, water, CO_2 and mineral salts for growth. The N_2-fixing species thrive without combined nitrogen and have, therefore, the simplest nutrient requirements among all organisms. They are often abundant in nature, although the reasons for their seasonal fluctuations and their sudden blooms are not fully understood. Photoproduction of H_2 catalyzed by hydrogenase has unambiguously been shown for the halophytic *Oscillatoria limnetica*[14]. The following findings show that H_2-formation by all other species is catalyzed solely by nitrogenase[7]: The addition of NH_4^+ to the cultures obliterates N_2-fixation (C_2H_2-reduction) and H_2-formation activities in a parallel fashion. Under aerobic conditions, H_2-formation is strictly light-dependent and sensitive to uncouplers

indicating the requirement for energy. Low activities are also observed in the dark when optimal amounts of O_2 are provided for the generation of ATP by respiration. H_2-formations are reduced by N_2 or C_2H_2 which compete with H_3O^+ for electrons in nitrogenase. Adaptation to anaerobic conditions is not necessary for H_2-evolution to begin.

N_2-fixation and thus H_2-formation is found in unicellular, filamentous non-heterocystous and in filamentous, heterocystous species[5]. Unicellular forms are the coenobial *Gloeocapsa* (now designated as *Gloeothece*) and *Aphanothece* which are slow-growing algae. A number of filamentous, non-heterocystous forms (e.g. *Plectonema boryanum*) perform N_2-fixation under very low O_2-tensions. The best-known examples for N_2-fixing blue-green algae are *Anabaena* species and *Nostoc muscorum*. These filamentous forms contain 2 cell-types, the vegetative cells and the heterocysts (fig. 2). The vegetative cells perform photosynthetic CO_2-fixation and O_2-evolution and provide the heterocysts with fixed carbon compounds. Heterocysts lack the photosynthetic water splitting reaction and are therefore not exposed to O_2 produced photosynthetically. Under aerobic conditions, nitrogenase was shown to be located exclusively in these specialized cells. This means that nitrogenase must be protected against damage by O_2 diffusing into the heterocysts. The protection mechanisms have not yet been fully elucidated, although respiration and the oxyhydrogen reaction seemingly are of major importance in removing O_2 from the nitrogenase site.

III. *The extent of H_2-production by blue-green algae*

In our own experiments, always very small amounts of H_2 were produced by aerobically grown blue-green algae (*Anabaena* species, *Nostoc muscorum*) assayed either aerobically or under strict O_2-exclusion and under limiting or saturating light conditions[7]. Maximal rates were approximately 1% of those obtained for photosynthetic CO_2-fixation and 10% of the C_2H_2-reduction capability. H_2-formation was increased to some extent by incubating the cells with optimal concentrations of CO and C_2H_2 which block the reutilization of H_2 by the oxyhydrogen reaction and hydrogenase (see section Ic and Bothe et al.[15]). Higher rates of H_2-evolution can also be obtained by artificially increasing the number of heterocysts. This can be achieved by treating the cultures with 7-azatryptophan[16]. However, maximal H_2-production never exceeded $\frac{1}{5}$ of the rate of C_2H_2-reduction. Other researchers[17-19], too, were unable to obtain high H_2-formation rates by blue-green algae. In a more systematic survey, Berchtold and Bachofen[20] found only very little H_2-production by a whole series of new isolates from the Zürich area as well as by known laboratory strains under stationary conditions. In contrast, a number of investigators reported high and long lasting H_2-productions when the gasses formed (H_2 and O_2) are constantly removed[21-24]. The absolute maximum is probably the 7500 µl H_2 produced/h × mg chlorophyll for *Anabaena cylindrica*[20]. Unfortunately, any experimental detail for such a high value is missing in this publication. Using severely nitrogen starved *Anabaena*, Weissman and Benemann[21] and Jeffries et al.[22] reported long lasting H_2-formations where the ratio between H_2-formation and photosynthetic O_2-evolution approached one. The efficiency of converting solar energy to H_2 was maximally 0.4%[21] or ranged between 0.35 and 0.85%[22] and the cells produced H_2 up to 19 or 30 days, respectively. A marine, non-bacteria free strain was able to evolve 250 µmoles H_2/h × mg chlorophyll[25].

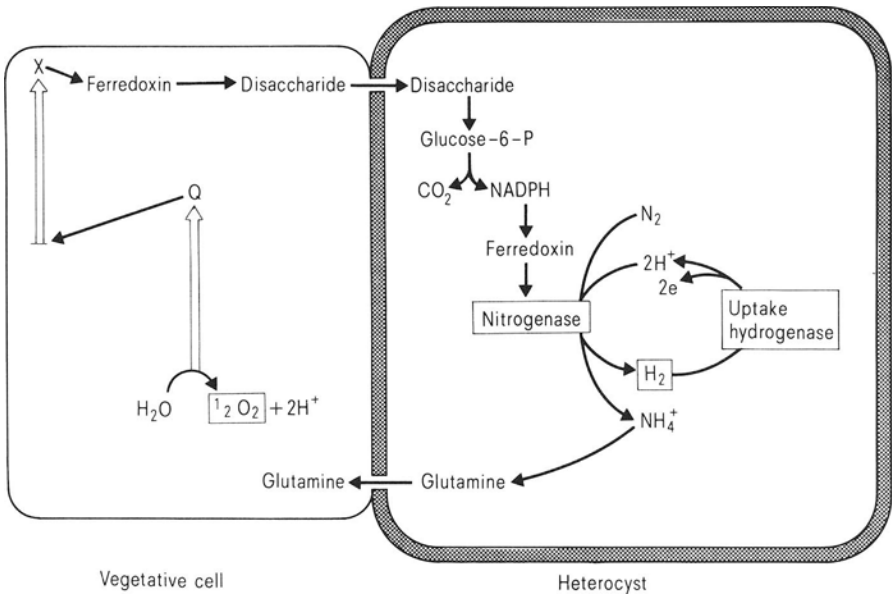

Figure 2. Metabolism of photosynthesis in vegetative cells and of nitrogen fixation, hydrogen production and hydrogen uptake in heterocysts of blue green algae.

It is difficult to judge the reliability of all these divergent data. It has become clear, however, that H_2-formation rates depend not so much on the algal strain used but on the culture and assay conditions employed. A major factor influencing H_2-formation capability is the nitrogen content of the cells. Prolonged nitrogen starvation, achieved by incubating the algae under argon in the absence of combined nitrogen for days, leads to the synthesis of additional nitrogenase (probably at the expense of phycocyanin) and increases the C_2H_2-reduction[26] activities and H_2-formation[21] capabilities. Also inhibition of the oxyhydrogen reaction by the uptake hydrogenase is prerequisital for a high H_2-production[15,27]. Incubation of *Nostoc* under an atmosphere of argon plus C_2H_2 has recently been shown to enhance H_2-formation considerably[23]. H_2-production is also influenced by other factors. These include during growth: the temperature, the supply of the culture with iron and CO_2, the O_2 tensions and the light intensities. During the assays rates largely depend on the duration of the experiments, the concentration of O_2, N_2 and CO_2 and of algal cells in the vessels and on the nutritional status of the cells, particularly on their reserves of organic carbon and nitrogen.

Obviously the optimal physiological conditions for maximal H_2-production have not yet been established. It must be pointed out that H_2-formation capability is limited by the nitrogenase content of the cells. With the strains commonly used in the laboratories, C_2H_2-reductions (as a measure of nitrogenase activity) vary between 5 and 20 μmoles C_2H_4 formed/h × mg chlorophyll. This is $\frac{1}{5}$ to $\frac{1}{10}$ of the rate of photosynthetic CO_2-fixation, in accord with the requirements of the cells for fixed carbon and nitrogen compounds. A rate of 20 μmoles H_2 formed/h × mg chlorophyll (~ 400 ml H_2/h × g chlorophyll = ~ 10 ml/h × g dry weight) means that all the electrons flowing to nitrogenase must reduce H^+. Only cells devoid of any regulatory mechanism to switch off the energy consuming reaction would sustain such high production over a longer period. Then they would have to be supplied with combined nitrogen. The addition of combined nitrogen (ammonia or nitrate), however, represses the synthesis of new nitrogenase molecules which should cause a gradual decline in the H_2-formations. To conclude, I am not convinced about H_2-productions which account to 20 or more, μmoles/h × mg chlorophyll and which last over a longer period.

On the other hand, autotrophic blue-green algae clearly have the potential to produce H_2 in a light-dependent reaction. We are only beginning to understand the physiology of the process. Basic research is necessary to find out the maximal capability for H_2-production and solar energy conversion. The search for new strains may be rewarding. The blue-green algae commonly used in the laboratory are probably not the fastest growing strains. The newly isolated *Anabaena CA*[28] and *Anabaena TA 1*[29] show considerably shorter generation times and higher N_2-fixation activities and are promising candidates for further investigation of H_2-formation. Genetic manipulations may be rewarding. Mutants that lack the uptake hydrogenase could be selected. N_2-fixation and consequently H_2-formation rates exceeding 20 μmoles H_2/h × mg chlorophyll would require additional amounts of reductant and ATP which would have to be provided by photosynthesis. Mutants could possibly be constructed which have higher photosynthetic capabilities or which furnish carbohydrates to heterocysts with higher rates but still survive. It may take a long time until a fair judgement can be made on whether blue-green algae are of value in projects of solar energy conversion programs.

In the near future, additional investigation of the hydrogenase-nitrogenase relationship is conceivable[30]. *Rhizobium* strains that possess the uptake hydrogenase were shown to fix N_2 more efficiently and to grow faster than strains without the enzyme. In nature, *Rhizobia* of the nodules of leguminous plants often do not possess an active hydrogenase. The productivity of plants could be improved by the applications of newly constructed *Rhizobium*-legume symbioses. These should have an active uptake hydrogenase and would have to compete with those currently existing in nature. Such projects could help save energy in a more indirect way.

Notes added in proof. Kayano et al.[33] have now immobilized an *Anabaena* strain in 2% agar gel where it produces H_2 with higher rates (≤ 0.5 μmoles/h × g gel) than in the free state. A photo-current of 15–20 mA was continuously produced for 7 days by a photochemical system consisting of the immobilized *Anabaena*, an oxygen removing 'reactor' containing aerobic bacteria and a hydrogen-oxygen fuel cell[33].

Hallenbeck et al.[34] have reported H_2-formation catalyzed by *Anabaena*. Their maximal rate (~ 2 μmoles/h × g dry weight, see table 1 in their paper) is self-evident. Any uptake hydrogenase should catalyze the reverse reaction at such an extremely low rate. Alternatively, bacterial contaminations producing these quantities of H_2 are difficult to dismiss in such experiments with large scale batch cultures. Houchins and Burris[35,36] have separated and biochemically characterized a reversible, soluble hydrogenase and the uptake hydrogenase from blue-green algae. Much of their data can not be simply reconciled with our own findings. Definitive proof for the existence of two different hydrogenases can only come from immunological studies. An excellent detailed account on hydrogenase by microorganisms has now been published by Mortenson's group[37].

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Hydrogen production by photosynthetic bacteria

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Photosynthetic bacteria utilize hydrogen as electron donor for autotrophic CO_2 assimilation. Many of these organisms also evolve hydrogen under dark anaerobic conditions and, in large quantities, anaerobically in the light in the absence of ammonia and molecular nitrogen. Hydrogen photoproduction in photosynthetic bacteria is largely or completely associated with the action of nitrogenase. It is not inhibited by CO, an inhibitor of hydrogenase and is dependent on ATP. The conventional hydrogenase catalyzes the reversible reaction $H_2 \rightleftarrows 2\ H^+ + 2\ e^-$. It seems however that in photosynthetic bacteria this enzyme catalyzes mainly hydrogen uptake in vivo. It has been suggested that a function of hydrogenase is to reutilize the hydrogen which is evolved as a byproduct of the nitrogenase reaction, retaining reducing equivalents for N_2 or CO_2 reduction[1]. In contrast to aerobic bacteria, energy conservation in a Knallgas reaction is not possible for photosynthetic bacteria growing anaerobically in the light[2]. Besides molecular hydrogen, a variety of organic and inorganic electron donors are known in bacterial photosynthesis. Most of them are effective also for hydrogen production in the light.

Hydrogen production and utilization in vivo are catalyzed by different enzymes. A genetic or regulatory linkage between nitrogenase and hydrogenase has been proposed in a study with nif^- mutants of *Rhodopseudomonas acidophila*[2]. It has recently been reported that in *Rhodopseudomonas capsulata* although nitrogenase may influence hydrogenase synthesis by supplying inducers (e.g., H_2), there is no strict correlation between hydrogenase synthesis and nitrogenase synthesis[3].

The exact mechanism of electron transfer in hydrogen metabolism and nitrogen fixation is not resolved so far. The figure shows a possible scheme of electron transport and hydrogen metabolism in the photosynthetic bacterium *Rhodospirillum rubrum*. A light driven electron flow generates ATP. It is assumed that NAD and other substances of negative redoxpotential are reduced in a reversed electron flow utilizing ATP.