The Gas Exchange of Hydrogen-adapted Algae as Followed by Mass Spectrometry

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ABSTRACT

A mass spectrometer inlet and an oxygen electrode in the same vessel allowed the continuous recording of the gas exchanged (H\textsubscript{2}, CO\textsubscript{2}, O\textsubscript{2}) by hydrogenase-containing anaerobically adapted Scenedesmus obliquus strain D\textsubscript{2} (Gaffron) and Chlorella fusca Shihira et Krauss (= pyrenoidosa) 211-15. A light intensity which produces more photosynthetic oxygen than the cells can re-reduce to water leads to de-adaptation and the substitution of normal photosynthesis for photoreduction. The sequence of these metabolic events was recorded in a matter of a few minutes. Upon exposure of these adapted algae to light, an evolution of hydrogen lasting up to 60 seconds preceded any other light-dependent gas exchange. In the presence of 3-(3,4-dichlorophenyl)-1,1-dimethyleurea, this initial hydrogen production was inhibited approximately 50%, pointing to a contribution of electrons by photosystem II. At very low hydrogen tensions (0.1 microliter per milliliter), a balance between light-induced production and absorption of hydrogen was observed in normal, unpolluted algae. Addition of either glucose or inhibitors of phosphorylation increased the release of hydrogen in the light very considerably. When the light was turned off the algae consumed the remaining amount of hydrogen, only to release it again upon illumination. This reversible hydrogen exchange persisted even when any concomitant carbon dioxide exchange had been abolished.

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\begin{align*}
\text{CO}_2 + 4\text{H}_2\text{O} + \sim \text{P} & \rightarrow (\text{CH}_2\text{O}) + \text{O}_2 + 3\text{H}_2\text{O} \\
\text{Photoreduction: } \text{CO}_2 + 2\text{H}_2 + \sim \text{P} & \xrightarrow{\text{H}_2\text{ase}} (\text{CH}_2\text{O}) + \text{H}_2\text{O} \\
\text{H}_2 \text{ photoproduction: } \text{RH}_2 & \xrightarrow{\text{H}_2\text{ase}} \text{R} + \text{H}_2 \\
\text{Dark enzymatic reactions:} & \\
\text{Respiration: } \frac{1}{2} \text{O}_2 + \text{RH}_2 & \rightarrow \text{H}_2\text{O} + \text{R} + \sim \text{P} \\
\text{Dark H}_2 \text{ production: } \text{RH}_2 & \xrightarrow{\text{H}_2\text{ase}} \text{R} + \text{H}_2 \\
\text{H}_2 \text{ absorption: } \text{H}_2 + \text{R} & \xrightarrow{\text{H}_2\text{ase}} \text{RH}_2 \\
\text{Oxy-hydrogen reaction: } \text{O}_2 + 2\text{H}_2 & \xrightarrow{\text{H}_2\text{ase}} 2\text{H}_2\text{O} + \sim \text{P} \\
\text{CO}_2 + 2\text{H}_2 & + \sim \text{P} \xrightarrow{\text{H}_2\text{ase}} (\text{CH}_2\text{O}) + \text{H}_2\text{O} \\
\end{align*}
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Without phosphorylation (e.g., in the presence of certain inhibitors) all energy-requiring synthetic processes (reactions 1, 2, 7b) come to a halt. Under such circumstances the light-dependent evolution of hydrogen (reaction 3) becomes conspicuous and long lasting (21, 24).

Our sensitive method of analyzing the simultaneous interplay of the gas exchanges of CO\textsubscript{2}, H\textsubscript{2}, and O\textsubscript{2} shows that hydrogen production precedes all other light effects listed above, and hence is more directly connected with the functions of the pigment system in algae.

MATERIALS AND METHODS

Autotrophic cultures of Scenedesmus obliquus strain D\textsubscript{2} (Gaffron) and Chlorella fusca Shihira et Krauss (= pyrenoidosa) 211-15 were grown as described previously (16). Heterotrophic and photoheterotrophic cultures, grown under sterile conditions in screw capped Erlenmeyer flasks in the same inorganic medium supplemented with 0.5% glucose and 0.025% yeast extract, were placed on a rotary shaker in darkness (heterotrophic cells) or in dim light (4.6 \times 10^7 \mu W cm\textsuperscript{-2}, photoheterotrophic cells).

After harvesting the cells as described previously (16), 0.4-ml packed cell volume was suspended in 120 ml of either 66 mm sodium phosphate buffer, pH 6.5, or 66 mm sodium phosphate-citrate buffer, pH 4.6, and stirred magnetically under a slow stream of H\textsubscript{2} in 200-ml round flasks. The lower pH was used in experiments involving the measurement of CO\textsubscript{2}, since this compound is partially hydrated at higher pH values. After adapting the cells overnight at room temperature, samples were removed with a syringe needle inserted through a rubber sleeve serum stopper.

The anaerobic sample was immediately transferred to the O\textsubscript{2}-free mass spectrometer inlet chamber (volume 6.0 ml). The design of the chamber is such that samples can be introduced

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2 Abbreviations: CI-CCP: carbonyl cyanide m-chlorophenylhydrazone; H\textsubscript{2}\text{ase}: hydrogenase; \sim \text{P}: high energy phosphate; PS: photosystem; R: unknown organic compounds (R: oxidized form; RH\textsubscript{2}: reduced form).
Plant with o-phenanthroline with the respiration. The sor). Hydrogenase 0.8 mately water (6)

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Fig. 1, A and B, are the first cases where the process of deadaptation in algae (specifically Scenedesmus obliquus in Fig. 1A and Chlorella fusca in Fig. 1B) has been recorded separately and in detail for \(H_2\), \(O_2\), and \(C_2\).

In the \(H_2\) recording we see no important changes of hydrogen partial pressures in the dark before illumination. At the end of the long anaerobic adaptation period, any enzymatic uptake of \(H_2\) in the dark had almost ceased. The absence of a change in the \(C_2\) concentration indicates that fermentative reactions had also become negligible.

Upon illumination, the cells slowly began to fix \(C_2\) via two simultaneous reactions, namely photoreduction with \(H_2\) and normal photosynthesis. As photosynthesis continued, the accumulated \(O_2\) progressively inhibited the reactions with \(H_2\). The hydrogenase was already partially inhibited by approximately 0.8 \(\mu\)mol \(O_2/\text{ml}\) and was totally inhibited by roughly 2.6 \(\mu\)mol \(O_2/\text{ml}\) (i.e., about 9% of \(O_2\) saturation). Upon darkening, the accumulated \(O_2\) was consumed (and \(C_2\) was produced) by normal respiration. No hydrogen metabolism was found during the second dark period due to the inactivation of the hydrogenase.

All of this has been known for many years (5, 8, 14, 15, 20).

What emerges as particularly striking in the mass spectrometer recordings is the initial photoevolution of \(H_2\). During the first minute of light, only \(H_2\) photoproduction was recorded, while photoreduction and photosynthesis showed induction periods. The induction period for photoreduction was evidently not due to the reduction of \(C_2\) by endogenous H-donors (cf. 6), since the \(C_2\) concentration did not change during this period. In agreement with an earlier manometric study (9) and these results, Spruit (20) found with a rapid polarographic method that adapted cells of Chlorella vulgaris show an immediate "burst" of \(H_2\) upon illumination. However, he also reported that the evolved \(H_2\) was accompanied by \(O_2\). It is not yet clear whether this discrepancy is merely due to the different experimental conditions employed in the two studies or reflects fundamental differences in the behavior of the algal species. In any case, the initial gush of \(H_2\) is probably part of the normal photochemistry of adaptable green algae. In the following we shall describe this initial reaction in more detail.

Elimination of Photosystem II Activity. A deadaptation of the hydrogenase may be avoided either by keeping the light intensity so low that whatever \(O_2\) appears is reduced back to water (6) or by inhibiting \(O_2\) evolution either by mutation (3) or with specific poisons which still permit photoreduction (e.g., \(o\)-phenanthroline [7]; DCMU [1]).

Figure 2 compares the course of \(H_2\) and \(O_2\) gas exchange in normal and DCMU-poisoned Scenedesmus. Here the first light period lasted only a little over 2 min, and the beginning of a second light period is shown which followed 2 min of darkness. The traces produced by the \(O_2\) electrode recorder show that adding DCMU prevented any measurable evolution of \(O_2\), while photosynthesis in the unpoisoned algae started without more than the short induction delay, despite complete anaerobiosis. (Compare the extensive discussion in the earlier literature on whether traces of \(O_2\) are needed for the initiation of photosynthesis [8].) Because 2 min instead of 10 min of light (Fig. 1) did not produce sufficient \(O_2\) for a complete de-
activation of the hydrogenase, one can see in Figure 2 a strong oxy-hydrogen reaction during the following dark period for the sample without DCMU. Poisoning with DCMU had two consequences. The initial H2 gush at “light on” as seen in Figure 1, A and B, was cut to less than half its size, and further, since no O2 was produced, the oxy-hydrogen reaction did not appear. This effect of DCMU on the initial H2 gush corresponds with those of our earlier experiments which have led us to assume that not only PS I but also PS II can deliver electrons to the hydrogenase system (21, 22, and unpublished results). In Figure 2, once the gas exchange has gone beyond the first induction periods, the rest of the two recordings of hydrogen uptake in the light are not as different as they appear. In agreement with Bishop (3), the apparent inhibition of photo-reduction seen in the presence of 10 μM DCMU was perhaps due to the absence of the H2-consuming oxy-hydrogen reaction, since correcting the unpoisoned control for the dark oxy-hydrogen reaction gave about the same rate of H2 uptake as that found in the presence of DCMU. Further work is needed in this area, however, since there is some evidence that PS II may participate in photoreduction by Ankistrodesmus and Chlorella (10, 17, 18).

The effect of the 2 min of darkness on the shape of the induction seen at the second exposure to light is noteworthy. The distinct and extended evolution of hydrogen was not repeated and the induction period for photoreduction was much shorter. The absence of a measurable photoproduction of H2 after this short dark period may be due to the depletions of endogenous H-donors and/or the masking of this reaction by rapid H2 consumption.

The Balance between Production and Utilization of Hydrogen. Figure 3 shows the stepwise depletion of H2 in the solution by photoreducing algae. These algae had been stabilized against deadadaptation with 10 μM DCMU, but no other inhibitor was present. The cells had been subjected to several periods of illumination before this part of the record was made.

In contrast to measurements made with DCMU-poisoned cells at high partial pressures (Fig. 2), an initial burst of H2 photoproduction was found (even after short dark periods) when such cells were illuminated at low partial pressures (Fig. 3). It is possible that this “burst” was not detected at higher partial pressures of H2 simply because the sensitivity of our instrument was enhanced at lower H2 concentrations (23).

The uptake of H2 via photoreduction stopped fairly abruptly at about 0.1 μl H2/mi even though the illumination continued. From there on no H2 exchange could be seen until the cells were darkened, after which they took up the remaining H2. When at zero H2 partial pressure the light was turned on for the third time, the expected evolution of H2 continued until the previous light level of about 0.1 μl H2/cm2 was reached (the “overshoot” found after longer dark periods will be discussed in another publication). This switching back and forth between no H2 at all and a new balance may be repeated many times.

![Figure 2](image1.png)  
**Fig. 2.** Effect of DCMU upon the initial gas exchange of adapted algae. Autotrophically grown cells (40 μl) adapted as described in “Materials and Methods,” in 60 ml of 66 mM sodium phosphate buffer, pH 6.5, flushed with 5% CO2 in H2. DCMU (final concentration 10 μM) was added 10 min before illumination (3.7 × 104 μw cm-2). Other experimental conditions as in Figure 1.

![Figure 3](image2.png)  
**Fig. 3.** The “balance” between H2 production and H2 consumption found at low H2 partial pressures. Forty μl autotrophically grown Scenedesmus, adapted as described in “Materials and Methods,” in 60 mM sodium phosphate buffer, pH 6.5, flushed with 5% CO2 in H2. DCMU (final concentration 10 μM) was added 10 min before illumination (1.5 × 104 μw cm-2) in order to prevent O2 evolution and deadaptation. Other experimental conditions as in Figure 1.
times. A similar balance between photoreduction and \( \text{H}_2 \) photoproduction was found for heterotrophically and photoheterotrophically grown cells of *Scenedesmus obliquus* and autotrophically grown *Chlorella fusca*.

If the "balance" is due to simultaneous photoreduction and \( \text{H}_2 \) photoproduction, any interference with the former reaction should disrupt the balance in favor of \( \text{H}_2 \) evolution. Figures 4 and 5 do indeed show that \( \text{H}_2 \) photoproduction was strongly enhanced when photoreduction was inhibited with CI-CCP, salicylaldoxime or glucose. CI-CCP and salicylaldoxime are both potent inhibitors of photophosphorylation (12, 13, 21). Glucose, in contrast, apparently interferes with photoreduction by competing for photochemically produced ATP (2). This compound also, at least in *Scenedesmus*, may contribute electrons for \( \text{H}_2 \) photoproduction (9, 22).

Since the rate of \( \text{H}_2 \) evolution always decreased rapidly with time, measuring meaningful rates was somewhat difficult. However, when interference from other reactions was eliminated with inhibitors (Figs. 4, 5), the initial rate of \( \text{H}_2 \) production was apparently quite high. For example, Figure 5 shows initial rates for \( \text{H}_2 \) production of about 60 \( \mu \)moles \( \text{H}_2/\text{mg chlorophyll} \) or about twice the rate of photosynthesis or photoreduction found under these experimental conditions (Fig. 1). Measurements of the quantum requirement for these three reactions are planned.

Figures 4 and 5 also show that the \( \text{H}_2 \) evolved in the light was reabsorbed in the dark. The rate of absorption decreased with time. Upon illumination, the initial rate of \( \text{H}_2 \) photoproduction was enhanced, but soon returned to the rate found before the dark period (cf. Fig. 4B). Under these experimental conditions, about half of the \( \text{H}_2 \) absorbed during the dark period could be quickly reutilized for \( \text{H}_2 \) photoproduction.

When photoreduction was inhibited with salicylaldoxime, light had little if any effect on changes in the concentration of \( \text{CO}_2 \), yet \( \text{H}_2 \) production continued as a strictly light-dependent reaction (Fig. 6). Although adapted cells slowly produce both \( \text{H}_2 \) and \( \text{CO}_2 \) (9, 11, 16, 25), it remains doubtful, at least in *Scenedesmus obliquus* and *Chlorella fusca*, that there is any direct connection between these two processes.

### DISCUSSION

The experiments with our mass spectrometer confirm the known over-all kinetics of the light-induced \( \text{H}_2 \) exchange by adapted algae. However, we could make three new and unexpected observations. First, after anaerobic adaptation in the dark, the initial photochemical response to illumination was always an evolution of \( \text{H}_2 \) which was not accompanied by a corresponding change in the partial pressure of \( \text{O}_2 \) or \( \text{CO}_2 \). When both \( \text{H}_2 \) and \( \text{CO}_2 \) were present, this visible "hydrogen gush" soon disappeared, and the cells began to consume \( \text{H}_2 \) via photoreduction. The photoreduction of \( \text{CO}_2 \) always showed an induction period, presumably because after a long dark period new \( \text{CO}_2 \) acceptors had to be provided by phosphorylation.

Second, when deadaptation was prevented by the addition of DCMU, without stopping photoreduction, the latter reaction could not deplete the experimental vessel completely of the last traces of free \( \text{H}_2 \). Instead, a balance resulted which evidently was due to a recycling of \( \text{H}_2 \) between photoproduction and photoreduction. Inhibitors of the latter reaction drastically disrupted the equilibrium in the light in favor of an accelerated and long lasting photoproduction of \( \text{H}_2 \). As described in an earlier paper (21), the treatment with salicylaldoxime isolated the photoproduction of hydrogen by PS I from the rest of the light metabolism in the algae.

The third observation was that under the conditions just described as a balance in the presence of DCMU, the algae in darkness were able to absorb all the remaining \( \text{H}_2 \). Our pre-
The experiments shown in Figures 3 to 6 thus strongly support the idea of a balance between the photoproduction and photoutilization of $\text{H}_2$. The ease with which this balance could be disturbed points to a mutually regulated, yet variable relationship between several energy-consuming photochemical reactions. The old question why the seemingly straightforward process of carbohydrate synthesis from hydrogen and $\text{CO}_2$ should have an unexpectedly high quantum requirement may find an explanation in these internal complexities (4).

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LITERATURE CITED