Proceedings of the 1999 U.S DOE Hydrogen Program Review
NREL/CP-570-26938

SUSTAINED PHOTOBIOLOGICAL HYDROGEN GAS PRODUCTION UPON REVERSIBLE INACTIVATION OF OXYGEN EVOLUTION IN THE GREEN ALGA *Chlamydomonas reinhardtii*

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Abstract

The work describes a novel approach for sustained photobiological production of H_2 gas *via* the *reversible hydrogenase* pathway in the green alga *Chlamydomonas reinhardtii*. This single-organism, two-stage H_2 production method circumvents the severe O_2-sensitivity of the reversible hydrogenase by temporally separating photosynthetic O_2 evolution and carbon accumulation from H_2 production in the culture. Following application of a stress condition (stress*; not identified in this report), photosystem-II function and O_2 evolution were reversibly inactivated in the chloroplast of this green alga. Oxidative respiration by the cells in the light generated anaerobiosis in the culture, a condition necessary and sufficient for the induction of the hydrogenase enzyme. This was followed by sustained cellular H_2 gas production in the light, but not in the dark. Rates of H_2 evolution, obtained with a Clark-type electrode, were about 22 mmol H_2 (mol Chl)$^{-1}$ s^{-1}, compared to 48 mmol O_2 (mol Chl)$^{-1}$ s^{-1}, measured prior to stress*. Collected volumes of H_2 gas were on the average 2 mL h^{-1}, compared to 12 mL O_2 h^{-1}, measured with the same culture in the

early stages of stress*. The mechanism for H_2 production entailed electron transport from endogenous substrate, through a chlororespiration-type process, to the cytochrome b_6-f and photosystem-I complexes in the chloroplast thylakoids. Light absorption by photosystem-I was an absolute prerequisite for H_2 evolution, suggesting that photoreduction of ferredoxin is followed by electron donation to the reversible hydrogenase. The latter catalyzes the reduction of protons to molecular H_2 in the chloroplast stroma.

Introduction

Interactions between molecular H_2 and living matter are widespread in nature. They are catalyzed by a diverse group of enzymes collectively known as 'hydrogenases' [Adams 1990, Albracht 1994]. Strategies of H_2 metabolism vary widely among the prokaryotic and eukaryotic organisms that undertake it [Hallenbeck and Benemann 1979, Weaver et al. 1980, Hall et al. 1995, Appel and Schulz 1998, Boichenko et al. 1999]. Hydrogen reactions can generally be divided into those that utilize the reducing power of H_2 to drive metabolic processes (H_2 consumption), and those that generate molecular H_2. In the first category, many photosynthetic and non-photosynthetic organisms can grow by using H_2 as the source of reductant [Weaver et al. 1980]. In the second category, reduction of protons by the enzyme 'hydrogenase' [Voordouw and Brenner 1985, Voordoux et al. 1989, Meyer and Gagnon 1991, Happe and Naber 1993, Peters et al. 1998] forms H_2 gas, which serves to dissipate excess 'electron pressure' within a cell. For example, anaerobic fermentative bacteria partially degrade organic carbon substrates to generate ATP. In the absence of an efficient electron sink (lack of O_2), some of these organisms use protons as a terminal electron-acceptor, thus releasing H_2 and permitting additional degradative steps in their metabolic pathway [Schlegel and Schneider 1978, Aoyama et al. 1997]. Under low partial pressures of molecular N_2, cyanobacterial heterocysts use reductant, supplied in the form of sugars by vegetative cells, and the enzyme nitrogenase [Benemann and Weare 1974, Houchins 1984, Hall et al. 1995] to generate H_2 from protons.

In eukaryotic algae, photosynthetic H_2 evolution has been detected transiently upon illumination [Gaffron and Rubin 1942], but only after a period of dark, anaerobic incubation of the culture that apparently "induces" the cell's ability to photo-produce H_2 [Roessler and Lien 1984, Happe et al. 1994, Ghirardi et al. 1997]. Photosynthetic H_2 evolution is accentuated under conditions of limiting CO_2, suggesting that the hydrogenase pathway operates in competition with the CO_2 fixation pathway in the consumption of chloroplast reductant [Kessler 1973, 1976]. Moreover, electron transport *via* the hydrogenase pathway is coupled to photosynthetic phosphorylation in the thylakoid membrane [Arnon et al. 1961], thus generating ATP which is essential for the maintenance and repair functions of the cell [Melis 1991].

Currently, photobiological production of H_2 by eukaryotic algae is of interest because it holds the promise of generating a renewable fuel from nature's most plentiful resources, light and water. Green algae, in particular, can utilize the energy of sunlight in photosynthesis to produce molecular H_2 from H_2O. In this photobiological H_2 production, electrons are extracted from water molecules on the oxidizing side of PSII. The potential energy of these electrons is elevated through utilization of absorbed sunlight in a step-wise manner, first at PSII and subsequently at

PSI. In this linear process of photosynthetic electron transport, electrons released upon the oxidation of water (E_{m7} = +820 mV) are eventually transported to the iron-sulfur protein ferredoxin (E_{m7} = -450 mV) on the reducing side of PSI. The so-called *reversible hydrogenase* in the stroma of the algal chloroplast (see below) accepts electrons from reduced ferredoxin and efficiently donates them to $2H^+$ to generate molecular H_2:

$$2H^+ + 2Fd^- \xleftarrow{\text{Hydrogenase}} H_2 + 2Fd \qquad (1)$$

Since the E_{m7} for H_2 oxidation is –420 mV and that for ferredoxin is –450 mV, it is thought that the equilibrium constant of the above reaction could be close to 1, hence the term 'reversible' assigned to the function of this hydrogenase.

Under the proper experimental conditions, i.e., purging of molecular O_2 from the reaction medium, absence of CO_2 and light-limited conditions, the quantum efficiency of the H_2 production process is close to the theoretical maximum and similar to that of oxygenic photosynthesis [Greenbaum 1982, 1988, Cinco et al. 1993]. The concept of "direct biophotolysis" [Benemann et al. 1973, Bishop et al. 1977, McBride et al. 1977, Weaver et al. 1980, Miura 1995] envisions light-driven simultaneous O_2 evolution on the oxidizing side of PSII and H_2 production on the reducing side of PSI, with a maximum H_2:O_2 (mol:mol) ratio of 2:1. Such a reaction with green algae could serve to provide a clean, renewable and economically viable H_2 fuel.

In practice, this potential has not as yet materialized because the reversible hydrogenase (i.e., the enzyme that combines protons and high-energy electrons into H_2) is extremely O_2 sensitive and is promptly deactivated at less than 2% O_2 partial pressure [Ghirardi et al. 1997]. An alternative approach to photoproduce H_2 is based on the concept of "indirect biophotolysis" in which starch accumulation acts as an intermediary electron carrier between photosynthetic H_2O oxidation and H_2 production. In this approach, the two reactions, O_2 evolution and H_2 production, are spatially and/or temporally separated from each other [Benemann 1996]. The present work describes the sustainable photosynthetic production of H_2 in a two-stage indirect biophotolysis process in which O_2 and H_2 production were temporally separated. This process of H_2 production operated continuously for several days.

Materials and Methods

Growth of the Algae

Chlamydomonas reinhardtii strain C137 (mt^+) was grown photoheterotrophically in a Tris-acetate-phosphate (TAP) medium, pH 7. Liquid cultures, bubbled with 3% CO_2 in air, were grown at 25°C in flat bottles (3-5 cm optical path length) upon stirring and under continuous cool-white fluorescence illumination at ~200 µmol of photons m^{-2} s^{-1}. Culture density was measured by cell counting with the improved Neubauer ultraplane hemacytometer and an Olympus BH-2 light microscope operated at a magnification of 200x.

Cells were grown to the late logarithmic phase (about 3-6x10^6 cells/mL). After they reached this density, cells were subjected to a stress* condition while incubated under continuous illumination for up to 150 h.

Oxygen Exchange and Hydrogen Evolution Measurements

At UC Berkeley, O_2 exchange activity of the cultures was measured at 25°C with a Clark-type O_2 electrode illuminated with a slide projector lamp. Yellow actinic excitation of saturating intensity was provided by a CS 3-69 Corning cut-off filter. A 5 mL aliquot of the culture was supplemented with 100 µL of 0.5 M $NaHCO_3$, pH 7.4 [Melis et al. 1999]. Measurements were taken with the O_2 electrode, beginning with the registration of dark respiration in the cell suspension, and followed by measurement of the light-saturated rate of O_2 evolution. The rate of each process was recorded for about 5 minutes. At NREL, rates of O_2 and H_2 evolution activity were measured with two different Clark-type electrodes, each poised for the optimal measurement of O_2 and H_2, respectively. Calibration of the electrodes was done as previously described [Seibert et al. 1998]. Saturating actinic illumination of about 1,300 µmol photons m^{-2} s^{-1} was provided by a Nolan-Jenner Model 170-D high intensity actinic source, filtered through a 1% $CuSO_4$ solution. Samples for H_2 evolution measurements were transferred from the culture bottle with argon-flushed gas-tight syringes into the argon-flushed Clark-type electrode chamber. The chamber was then bubbled with argon for ~3 min to remove H_2 dissolved into the growth medium. Rates of H_2 evolution were recorded upon illumination for 5 min. The H_2 concentration signal from the electrode was amplified with an in-line Ithaco Model 1201 amplifier, modified with a custom-built current-to-voltage converter and analyzed with a Data Translation DT31-EZ A/D data acquisition system using customized DTVee software. Photosynthetic O_2 evolution and oxidative respiration rates were measured as described above.

Gas Collection Measurements

Culture bottles (Schott or Roux type) were fitted with an #25 Ace thread and smaller side-ports for liquid sampling. A threaded glass stopper with capillaries for gas sampling was fitted with a Viton O-ring and used to seal the reactor. Threaded side-arm and gas sampling ports were sealed with rubber laminated Teflon septa. Teflon tubing (Aminco, HPLC), attached to one of the gas ports, was used to conduct gas evolved by the algae in the culture bottles to an upside-down graduated cylinder filled with H_2O. The gas collection tubing was detached from the culture bottle during liquid and gas sampling to avoid disturbance of gas volume readings in the graduated cylinder.

Determination of the Concentrations of CO_2 and H_2 in the Gas Phase

A Varian Model 3760 gas chromatograph with Varian Star 4.0 data analysis software was used to determine the levels of CO_2 and H_2 in the headspace of the reactor. A Supelco MS-5A molecular sieve column with argon as the carrier gas was used to separate O_2, N_2 and H_2. A Supelco Porapak Q column with He as the carrier gas was used to assay for CO_2. Signals were generated by the instrument's TC detector. Dissolved CO_2 was driven into the gas phase by

injection of the liquid sample into 2 N hydrochloric acid in an argon-flushed, septum-capped vial. The signals were calibrated by injection of known amounts of O_2, N_2, H_2 and CO_2.

Thylakoid Membrane Isolation and Analysis

Cells were harvested by centrifugation at 3,000xg for 3 min at 4°C. Pellets were diluted with sonication buffer containing 100 mM Tris-HCl (pH 6.8), 10 mM NaCl, 1 mM *p*-aminobenzamidine-2HCl, 1 mM 6-aminocaproic acid, 10 mM EDTA, and 100 µM PMSF. Cells were disrupted by sonication for 2 min in a Branson Sonifier (cell Disruptor 200) operated in the pulsed mode with a 50% duty cycle and an output power setting of 5. Unbroken cells and other large cell fragments were removed by centrifugation at 3,000xg for 3 min at 4°C. The supernatant was then centrifuged at 75,000xg for 30 min at 4°C. Chlorophyll (*a*+*b*) content of the samples was measured in 80% acetone by the method of Arnon [1949], with equations corrected as in Melis et al. [1987].

Spectrophotometric Measurements

The amplitude of the light *minus* dark absorbance difference measurements at 700 and 320 nm [Melis 1989] was employed for the direct quantitation of P700 and Q_A in the *C. reinhardtii* cultures. These measurements provided estimates of the concentration of functional PSI and PSII reaction centers, respectively [Melis 1989, 1991], in the samples at various times following initiation of the stress* condition. The amplitude of the hydroquinone-reduced *minus* ferricyanide-oxidized absorbance difference measurement at 554 nm, with isosbestic points at 544 and 560 nm, was employed in the quantitation of cytochrome *f*. Sample purification and preparation for these measurements were described earlier [Melis et al. 1996].

Acetate, Starch and Protein Quantitations

The levels of acetate were measured in the supernatant, following centrifugation of the algal cells at 1,000xg for 2 min. A Hewlett-Packard 1050 fully-integrated HPLC with a BioRad Aminex HPX-87H ion exchange column and UV-detector was used for these measurements. H_2SO_4 (4 mM) served as the mobile phase to separate organic acids. The output signals were analyzed with HP Chemstation software. Starch determinations were performed according to [Gfeller and Gibbs 1984] using amyloglucosidase (Sigma, St. Louis) to convert starch from methanol solubilized cells to glucose. The concentration of glucose was then determined using a D-Glucose test kit (Boehringer Mannheim). The test depends upon two enzymatic reactions, the phosphorylation of glucose to glucose 6-phosphate by hexokinase, and subsequent reduction of NAD^+ to NADH by glucose 6-phosphate. The amount of NADH accumulated was measured spectrophotometrically by determining the absorption change at 340 nm. Protein quantitation was implemented according to Lowry et al. [1951].

Results

Sustained Photobiological Production of Hydrogen Gas in *C. reinhardti*

When *Chlamydomonas reinhardtii* cultures are subjected to a stress condition (stress*), the light-saturated rates of O_2 evolution and CO_2 fixation decline significantly within 24 h in the light, without a proportional loss of chloroplast or thylakoid membrane electron transport components. Analysis indicated that such loss in electron transport activity is due to the conversion of PSII centers from the Q_B-reducing to a Q_B-nonreducing form. The result of stress* on photosynthesis and cellular respiration over a longer period of time (0-120 h) are shown in **Fig. 1**. The activity of photosynthesis, measured from the light-saturated rate of O_2 evolution in *Chlamydomonas reinhardtii* (Fig. 1, P), declined bi-exponentially as a function of time during the 0-120 h stress* period. Cellular respiration, measured from the rate of O_2 consumption in the dark (Fig. 1, R), remained fairly constant over the 0-70 h period and declined slightly thereafter. It is important to note that the absolute activity of photosynthesis decreased below the level of respiration in *Chlamydomonas reinhardtii* after about 24-30 h of stress*.

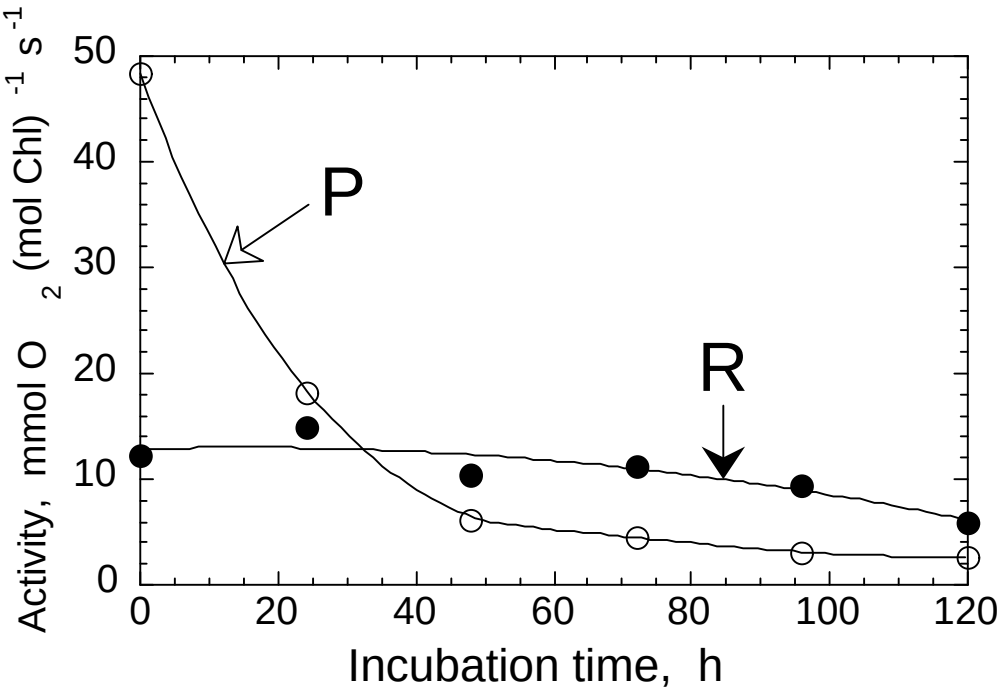

Figure 1 - Photosynthesis and Respiration in *C. reinhardtii*
Incubation under stress* started at 0 h. Cells were suspended in the presence of 10 mM $NaHCO_3$, pH 7.6. The rate of cellular respiration (R) was recorded in the dark, followed by a measurement of the rate of light-saturated photosynthesis. Net photosynthesis (P) was corrected for the rate of dark respiration.

We reasoned that, sometime after about 24-30 h of stress*, a sealed *Chlamydomonas reinhardtii* culture would quickly become anaerobic in the light, due to the significantly greater respiratory than photosynthetic activity of the cells. This was indeed confirmed by measurements with a Clark-type O_2 electrode (results not shown). Anaerobiosis is a necessary and sufficient condition for the induction of the reversible hydrogenase and light-induced H_2-production activity in *C. reinhardtii.* Conversely, as shown below, darkness *per se* is not an absolute prerequisite for the induction of the reversible hydrogenase and for a manifestation of its activity, provided that no O_2 is present. It was of particular interest, therefore, to test for light-dependent H_2 gas evolution and accumulation in stressed* *C. reinhardtii* cultures at times greater than 30 h.

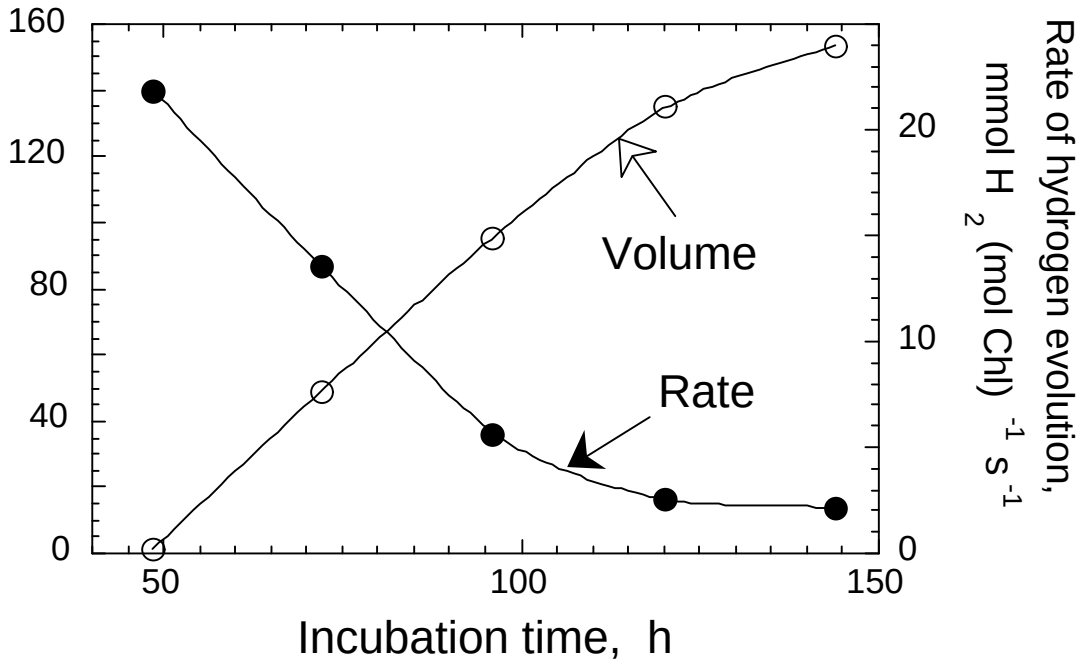

Figure 2 – Hydrogen Gas Production in *C. reinhardtii*
Hydrogen gas volume accumulated in a graduated cylinder (open circles) and the rate of light-saturated H_2 evolution measured with a Clark-type H_2 electrode (solid circles) as a function of incubation time under stress*.

Fig. 2 shows the result of such measurements, conducted at NREL with a stressed* culture of *C. reinhardtii*. In this experiment, a 1 L culture of algae at a cell density of about $6x10^6$ cells/mL was incubated under stress* and continuous illumination conditions. The culture bottle was sealed 43 h after application of stress*, when the rate of photosynthetic O_2 evolution was determined to be equal to or less than the rate of respiration. Subsequently, rates of H_2 evolution were measured in aliquots taken from the culture at 24 h intervals. The rate of H_2 evolution at time t=48 h was equal to 22 mmol H_2 $(mol\ Chl)^{-1}\ s^{-1}$ (Fig. 2, Rate). This rate is ~45% of the rate of O_2 evolution at t=0 h (see Fig. 1, P), but it is comparable to the rate of H_2 evolution measured with *C. reinhardtii* cells grown under photoautotrophic conditions [Seibert et al. 1998]. Thus, the stress* condition itself does not appear to exert a negative effect on the induction and activity

of the reversible hydrogenase at this point in time. However, the activity of this enzyme declined during subsequent incubation under stress*, reaching a low plateau of about 2 mmol H_2 (mol Chl)$^{-1}$ s^{-1} after 120 h.

Hydrogen gas accumulation was determined by measuring the amount of water that was displaced in an inverted graduated cylinder. The rate of gas accumulation was constant at ~2 mL h^{-1} (equivalent to 1.2 mmol H_2 [mol Chl]$^{-1}$ s^{-1}) for up to about 105 h. At this point the rate of accumulation started to level off (Fig. 2, Volume). Gas chromatographic analysis revealed that the composition of the headspace in the culture bottle at 120 h was about 87% H_2, 1% CO_2, with the remainder being N_2 and traces of O_2. A direct comparison of these results (Fig. 2, Rate *versus* Volume) suggests that, at t=48 h, *less than 10%* of the capacity for H_2 production (Fig. 2, Rate) is actually being utilized for the accumulation of H_2 gas in the graduated cylinder (Fig. 2, Volume) at this time point. At later times in this measurement (t>48 h), the rate of H_2 evolution declined (Fig. 2, Rate) and approached the rate of gas accumulation in the graduated cylinder (Fig. 2, Volume). It is clear from these results that factors other than the activity of the reversible hydrogenase are responsible for the slow H_2 gas accumulation, particularly in the early stages of this measurement.

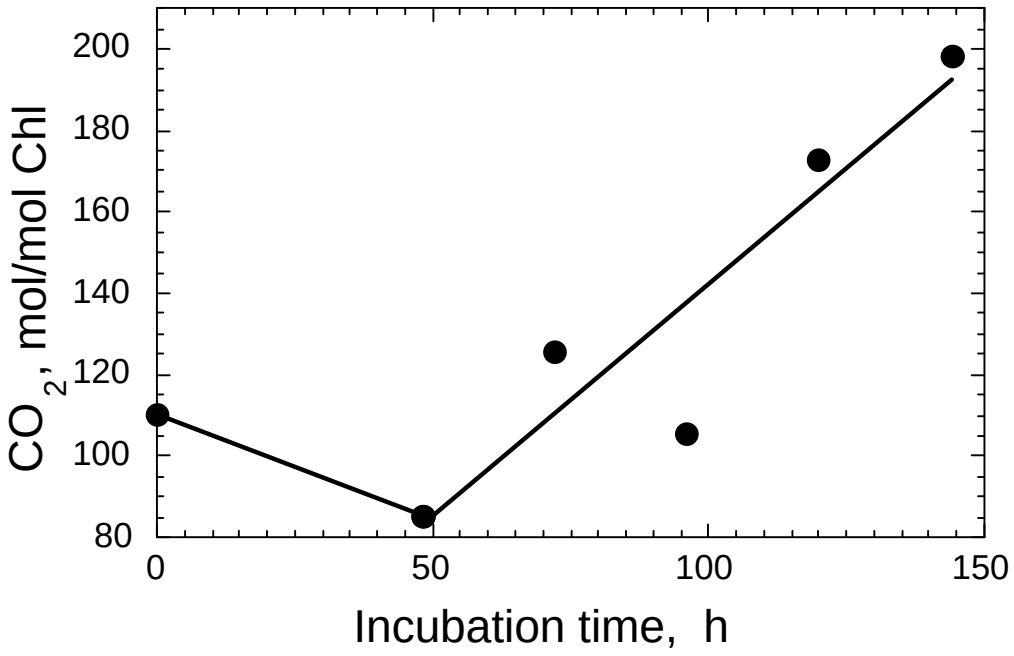

Figure 3 – Carbon Dioxide Gas Exchange in *C. reinhardtii*
Quantitation of dissolved CO_2 produced in tandem with H_2 by stressed* *C. reinhardtii* cultures. The culture was sealed at about 45 h following application of the stress* condition.

In addition to H_2, algal anaerobic photofermentations are expected to yield CO_2 and small amounts of formate and ethanol [Gfeller and Gibbs 1984]. **Fig. 3** shows that the amount of dissolved CO_2 declined during the 0-40 h period and, subsequently, increased in the 40-150 h

period from about 80 to about 200 mol CO_2 (mol Chl)$^{-1}$. This increase translated to a rate of 0.32 mmol CO_2 (mol Chl)$^{-1}$ s^{-1}. Given that under the same conditions we measured a rate of H_2 accumulation equal to 1.2 mmol H_2 (mol Chl)$^{-1}$ s^{-1}, we estimated a H_2/CO_2 (mol:mol) ratio of about 3.7:1 for this process. The amount of CO_2 in the headspace of the culture gradually increased from atmospheric values (0.03%) to about 1% in the course of the H_2 production period. This corresponds to a rate of CO_2 accumulation less than 0.5% of the rate of H_2 accumulation, and it is negligible compared to the amount of CO_2 that accumulated in the liquid phase. Furthermore, accumulation of fermentation byproducts, such as formate and ethanol, was not observed to occur in the growth medium during the course of the H_2 production period.

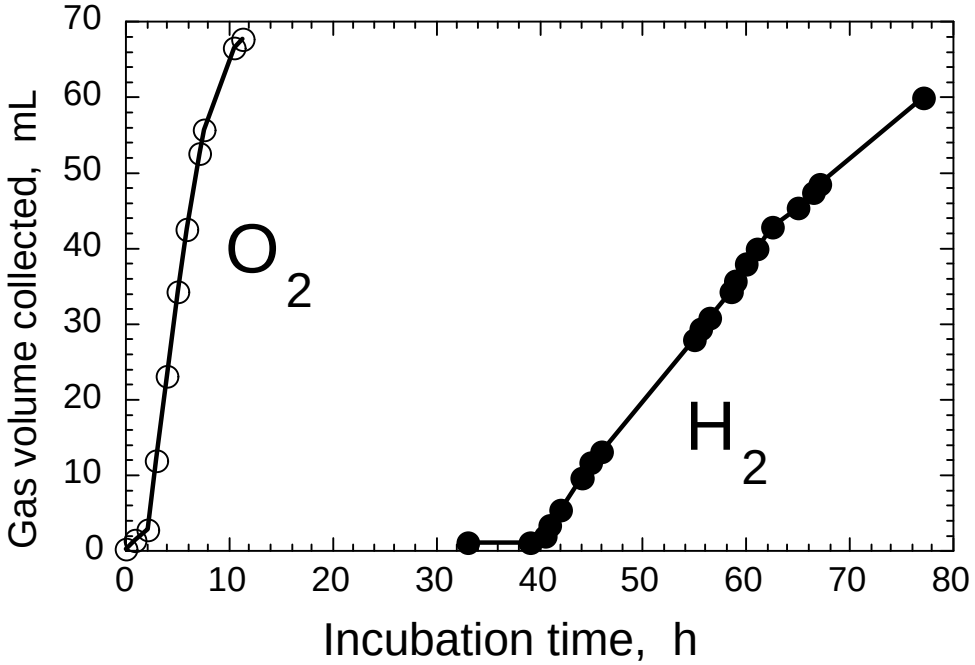

Figure 4 – Oxygen and Hydrogen Production in *C. reinhardtii*
Temporal separation of photosynthetic O_2 and H_2 gas production in cells under stress*. Gases were collected in an inverted graduated cylinder by displacement of water.

Fig. 4 shows the result of experiments, conducted at UC Berkeley, in which concomitant with the application of stress*, cultures were supplemented with 25 mM $NaHCO_3$, pH 7.6, to serve as the substrate of oxygenic photosynthesis. *C. reinhardtii* cultures grown in a Roux bottle (850 mL capacity), and having a density of about $3x10^6$ cells/mL, were incubated in the light. Cultures were sealed at 0 h and O_2 gas collection was measured with the inverted graduated cylinder setup. In such experiments, the maximum rate of O_2 gas accumulation (Fig. 4, O_2) was estimated to be about 12 mL O_2 h^{-1} (equivalent to 25 mmol O_2 (mol Chl)$^{-1}$ s^{-1}). This rate, not corrected for cellular respiration, is comparable to the average of the rates measured with a Clark-type O_2 electrode (Fig. 1, P). Hydrogen gas accumulation was measured with the same setup at later times, following the onset of anaerobiosis in the sealed cultures. The initial rate of H_2 gas accumulation (Fig. 4, H_2) was estimated to be about 2 mL H_2 h^{-1} (equivalent to 4.1 mmol H_2 (mol

Chl)$^{-1}$ s^{-1}), which, again, is significantly lower than the capacity for H_2 evolution, measured with the Clark-type electrode (Fig. 2, Rate). Furthermore, the rate of H_2 gas collection was less than 20% of the rate of O_2 collected in the inverted graduated cylinder (Fig. 4). The above results show a significant discrepancy in the estimates of the H_2:O_2 (mol:mol) ratio based on polarographic (Fig. 2, Rate) versus gas volumetric measurements (Fig. 2, Volume and Fig. 4, H_2). Polarographic measurements suggest a H_2:O_2=0.45:1 (mol:mol) ratio, whereas volumetric measurements showed only a H_2:O_2=0.17:1 (mol:mol) ratio. A discussion/analysis of these results is given below.

Structural and Functional Properties of the H_2-producing Photosynthetic Apparatus

The Chl content of the cells and the composition of the thylakoid membrane in *C. reinhardtii* changed upon application of the stress* condition. **Fig. 5** shows that the cell density of the culture increased transiently from about 3x10^6 cells/mL at 0 h to about 4x10^6 cells/mL at 60 h, and subsequently declined to 3x10^6 cells/mL at 120 h of stress*. Concomitantly, the Chl content of the culture declined steadily from about 8 μM to about 4 μM over the duration of this experiment.

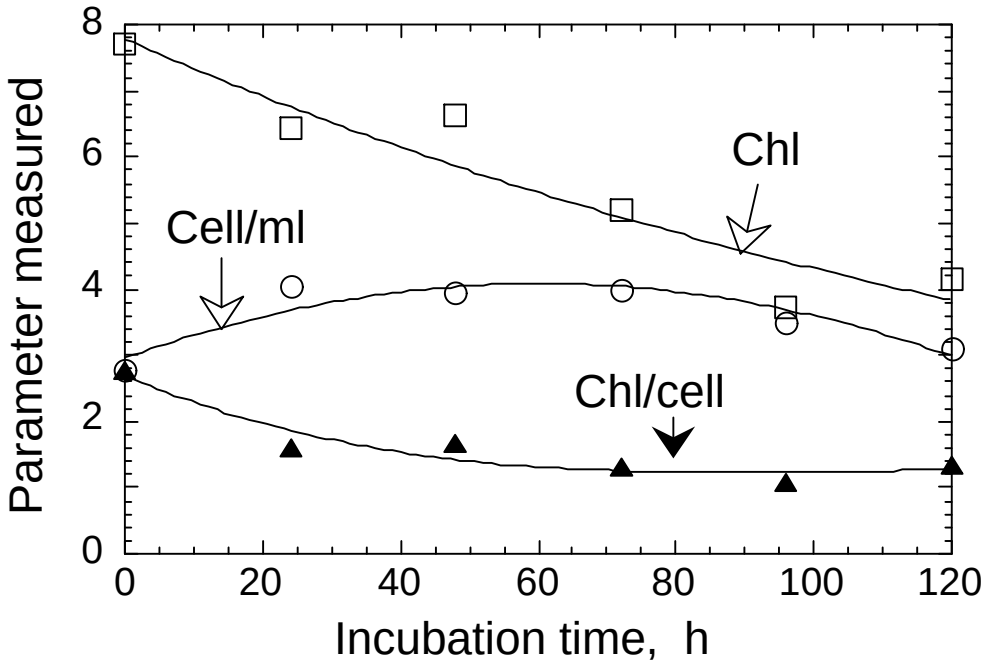

Figure 5 – Effect of Stress* on Cell Density and Chlorophyll Content
Chlorophyll concentration, cell density and chlorophyll content per cell in a *C. reinhardtii* culture under stress*. Initial values, at t=0 h, were Chl=7.7 μM, Cell/mL=2.8x10^6, Chl/cell=2.8x10^{-15} mol/cell.

The Chl content per cell declined from about $2.7x10^{-17}$ to about $1x10^{-17}$ mol Chl/cell following a 120 h incubation under stress*. These results show that some cell division does occur during the first 60 h of stress* but that a gradual loss of Chl also occurs throughout the deprivation period. Interestingly, the Chl *a*/Chl *b* ratio of the cells increased only slightly (from ~1.8:1 to 2.3:1) during the 0-120 h stress* period.

The concentration of integral thylakoid membrane complexes (PSII, Cyt b_6-*f* and PSI) in the thylakoid membrane of stressed* *C. reinhardtii* was investigated spectrophotometrically as follows: (1) from the amplitude of the light-*minus*-dark absorbance change at 320 nm (measuring the photochemical reduction of the primary quinone acceptor Q_A of PSII); (2) from the amplitude of the light-*minus*-dark absorbance change at 700 nm (measuring the photochemical oxidation of the reaction center P700 of PSI); and (3) from the hydroquinone-reduced *minus* ferricyanide-oxidized difference spectra of cytochrome *f* in isolated thylakoid membranes [Melis et al. 1996]. **Fig. 6** shows that the amount of all three functional components declined with incubation time, with PSII (Q_A) declining somewhat faster than P700 and Cyt *f*.

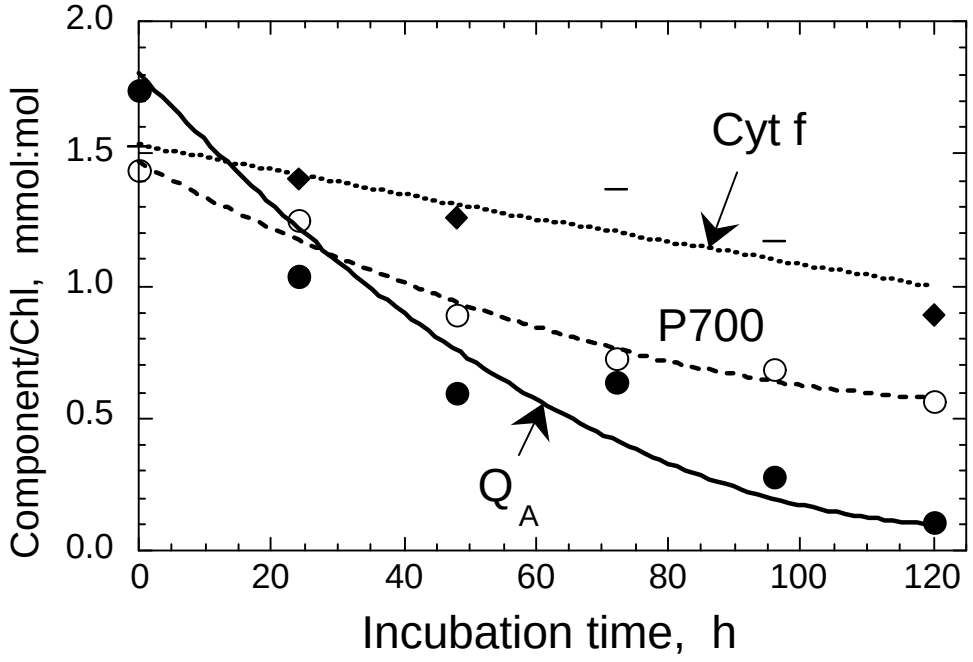

Figure 6 – Photochemical Apparatus Organization in *C. reinhardtii*
Concentration of functional PSII (Q_A), cytochrome b_6-*f* complex (Cyt *f*) and PSI (P700) as a function of time under stress*.

It is evident that loss of PSII centers that are functional in charge separation (Fig. 6, Q_A, half-time of 40 h) is considerably slower than the loss of O_2 evolution activity in the cells (Fig. 1, P, half-time of 20 h). These results are consistent with the notion that the stress* condition first causes a conversion of PSII centers from the Q_B-reducing to a Q_B-nonreducing form, followed by a slower loss of the PSII centers from the chloroplast thylakoids. This notion was supported by results of western blot analyses with antibodies specific for the various reaction center proteins of

PSII and PSI (not shown). Thus, the response of the cells to stress* suggests a strategy designed to prevent the generation of O_2, thus avoiding severe oxidative damage under stress*.

In the absence of functional PSII, the photobiological production of H_2 requires the presence and operation of PSI. Only PSI is capable of generating reduced intermediates (e.g., reduced ferredoxin) with a sufficiently negative midpoint redox potential for the generation of molecular H_2 [Redding et al. 1999]. Figure 6 (Cyt *f* and P700) shows that significant amounts of Cyt *f* and P700 are retained in the thylakoid membrane throughout the 120 h stress* period. Cyt b_6-*f* and PSI are needed for the transport of electrons from a chlororespiratory substrate ([Moller and Lin 1986], see also below) to ferredoxin and the reversible hydrogenase. A chlororespiration-supported PSI activity in this H_2 production process was shown by *in vivo* measurements of the photooxidation and recovery kinetics of P700 in stressed* cells that were suspended in the presence of the PSII electron transport inhibitor DCMU. **Fig. 7** shows such a kinetic trace in which actinic excitation (administered at 100 ms) caused a negative absorbance change at 700 nm (oxidation of P700 in the sample). When actinic excitation was turned off at 300 ms, P700 was reduced promptly in the dark with kinetics in the ms time range.

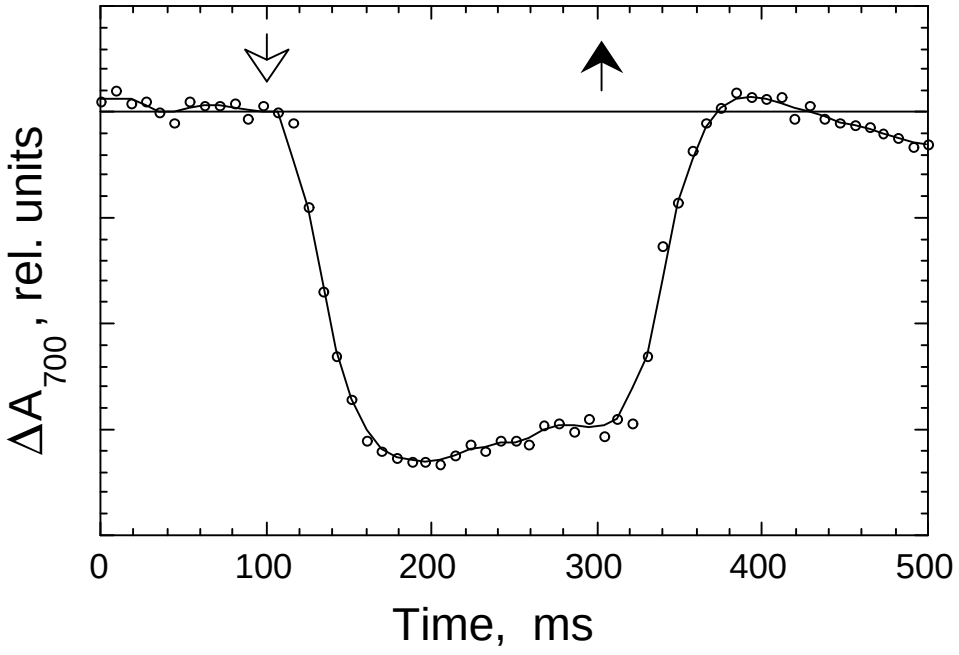

Figure 7 – Light-Induced Oxidation-Reduction Changes in *C. reinhardtii*
In vivo light-induced absorbance change measurements of P700 (ΔA_{700}) in stressed* *C. reinhardtii.* Cells were suspended in the presence of 20 μM DCMU. The time response of the apparatus was limited, through the use of electronic filters, to 15 ms. Saturating blue actinic excitation (CS 4-96 Corning glass filter, 250 μmol photons m^{-2} s^{-1}) came ON at 100 ms (open arrow) and went OFF at 300 ms (filled arrow).

The fast recovery of P700 in the dark suggests an abundance of electrons in the intersystem electron transport chain (plastoquinone, cytochrome b_6-*f* and plastocyanine).

Addition/omission of DCMU to the samples had no effect on the observed light-induced oxidation or dark recovery kinetics (results not shown), consistent with the absence of electron transport through PSII. This repetitive light-induced oxidation and dark-recovery pattern was kinetically identical in all samples examined throughout the 120 h stress* period, demonstrating the active operation of an electron-transport pathway that involves electron donation from a substrate to the thylakoid membrane of *C. reinhardtii*, probably at the level of the plastoquinone pool.

The role of various metabolites and the identity of the chlororespiratory substrate that serves as the source of electrons for this photobiological H_2 production were investigated. Acetate and starch are likely candidates for a chlororespiratory substrate in *C. reinhardtii* [Gibbs et al. 1986]. **Fig. 8 (Acetate)** shows that the amount of acetate in the culture medium declined by about 50% during the 0-30 h stress* period. However, it remained stable at this level during the 30-120 h period and even started to increase slightly thereafter (data points beyond 120 h not shown). These results suggest that acetate is consumed by respiration for as long as there is O_2 in the culture medium (0-30 h) but it does not contribute significantly to the source of electrons in the H_2-production process (30-120 h). Consistent with this interpretation are also measurements of the pH in the culture medium. The pH increased gradually (from 7.5 to 8.2) during the 0-30 h period of aerobic incubation under stress*, consistent with the uptake and utilization of acetate and the concomitant release of hydroxide anion as a by-product of this reaction.

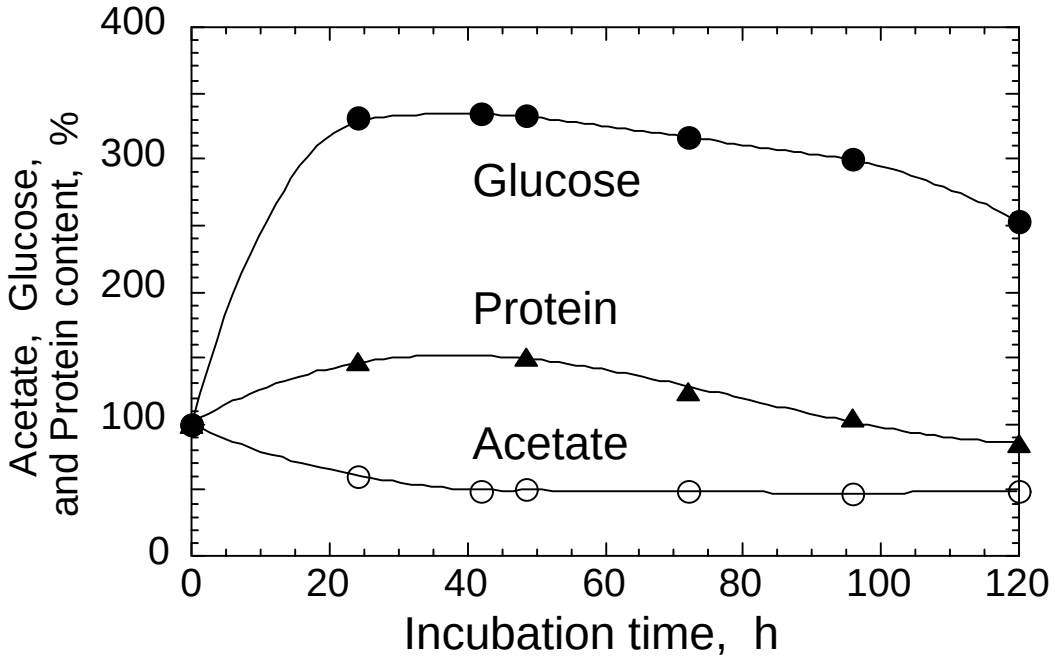

Figure 8 – Specific Metabolite Content in Cultures of *C. reinhardtii*
Acetate, protein and starch (measured as total glucose) contents in *C. reinhardtii* as a function of time under stress*. The absolute values at zero time were: acetate = 15 mM, starch = 16 nmol glucose/mL, and protein = 70 μg/mL.

Once anaerobiosis was established (t>30 h), however, this pH increase was gradually reversed (from ~8.2 to ~8.0), consistent with the notion of a light-dependent catabolic pathway that resulted in the formation of H_2 gas and CO_2. The majority of the released CO_2 was trapped in the culture medium as bicarbonate anion ($CO_2 + H_2O \rightarrow HCO_3^- + H^+$) due to the high pH value of the solution (see Fig. 3).

Interestingly, the amount of starch in the cells (measured as the total glucose content of the cells following starch hydrolysis), increased transiently by about 330% during the first 25 h of stress*, and subsequently declined to about 250% of the initial value at 120 h (**Fig. 8, Glucose**). The slow rate of glucose loss in the 25-120 h period suggests that starch catabolism is not sufficient to account for the electrons that feed into the reversible hydrogenase pathway (analysis not shown). Rather, cellular compounds such as protein and/or lipid, through their regulated catabolism in the cell, might be the source of the chlororespiratory substrate that provides reductant for the photobiological generation of molecular H_2. Indeed, quantitation of cellular protein in the stressed* cultures showed that the amount of protein increased transiently to about 150% of the initial in the 0-30 h period. Thereafter, in the 30-120 h period, and concomitant with the H_2 production activity, the level of protein in the culture declined to about 80% of the initial value (**Fig. 8, Protein**).

Discussion

The ability of green algae to produce H_2 directly from water has been recognized for over 55 years [Gaffron and Rubin 1942]. This activity is catalyzed by the reversible hydrogenase enzyme that is induced in the cells after exposure to a short, anaerobic period in the dark. However, the activity is rapidly lost as soon as the light is turned on, because of immediate deactivation of the reversible hydrogenase by photosynthetically-generated O_2. Although continuous purging of H_2-producing cultures with inert gases has allowed for the sustained production of H_2 for up to 160 h [Reeves and Greenbaum 1985], such purging is expensive and impractical for large-scale mass cultures of algae. The use of vacuum pumping, addition of exogenous reductants such as sodium dithionite, as well as the addition of herbicides to inhibit photosynthetic O_2 evolution is also problematic. Consequently, the absence of a physiological way of surmounting the O_2-sensitivity of hydrogenases has discouraged research on applied algal H_2-production systems. The results presented in this paper, however, show a novel method to temporally separate O_2- evolution and H_2-production activities, thus allowing H_2 production for extended periods of time without resorting to the use of the above-mentioned mechanical and chemical manipulations. The new method demonstrates, for the first time, the successful operation of a single-organism, two-stage photobiological H_2-production process in a green alga. The temporal sequence of events for the process is straightforward:

- The algae were grown in the light in a TAP medium until they reached a density of 3-6 million cells per mL in the culture (Stage 1).
- The cells were subjected to a stress* condition (Stage 2).
- The cultures continued to carry out oxygenic photosynthesis for about 30 h, until the absolute activity of photosynthesis gradually decreased to a level below that of respiration.

- The cultures were sealed and connected by Teflon tubing to a gas retrieval apparatus (in this case an inverted graduated cylinder filled with water and immersed in a water-containing beaker).
- Hydrogen gas, generated photobiologically, was collected in the graduate cylinder by displacement of water. The volume of the accumulating gas phase was measured directly in the graduate cylinder.
- Samples from the gas phase in the culture bottle, were removed with a syringe for gas chromatographic (GC) analysis. Aliquots of the liquid culture were also drawn for Clark-electrode, HPLC, enzymatic and spectrophotometric assays.

Why do *Chlamydomonas reinhardtii* cells produce molecular H_2 under these conditions? The most likely explanation is that H_2 evolution is the only mechanism available to the algae for generating sufficient amounts of ATP required for the continued survival of the organism. The main processes for ATP formation, mitochondrial respiration and oxygenic photosynthesis, are not available to sealed and stressed* *C. reinhardtii* cells due, respectively, to the lack of O_2 and inactivation of PSII function. Electron transport from a chlororespiratory substrate through the cytochrome b_6-f complex can generate the required ΔpH gradient across the thylakoid membrane for the generation of ATP. Electron transport from the Cyt b_6-f complex through PSI and the generation of molecular H_2 serves as the only way to sustain the electron transport process and ΔpH gradient formation, and it occurs at the expense of reductant that is eventually lost to the environment in the form of gaseous H_2. Perhaps cyclic electron transport around PSI, primed by electron donation through the chlororespiratory pathway also contributes to ATP production. Build-up of CO_2 as bicarbonate in the culture is another issue. It is possible that the decrease in H_2 production capacity as a function of time observed at greater than 50 h (Fig. 2) is due to increased electron transport partitioning away from hydrogenase and to the Benson-Calvin cycle.

The gradual inactivation of water-oxidation under stress* is an energy-dependent process, which requires light and/or a carbon substrate for respiration (data not shown). Our current experimental protocol uses both. The substrate for respiration in the initial 30 h of stress* is clearly acetate, as seen in Fig. 8. As the culture becomes anaerobic, acetate consumption by respiration decreases to zero. The H_2-production phase of the system is also a light-dependent process that utilizes the chlororespiratory pathway under anaerobic conditions. The fermentative metabolism of *C. reinhardtii* in the light was studied extensively by Gibbs et al. [Gfeller and Gibbs 1984, Gibbs et al. 1986, Maione and Gibbs 1986a,b]. The main products of the photofermentation of starch in the presence of DCMU (an inhibitor of PSII electron-transport and O_2 evolution whose addition is experimentally similar to the system describe here where PSII is inactivated) were found to be H_2 and CO_2, in a ratio of 2.8:1 (mol:mol) [Gfeller and Gibbs 1984]. Formate and ethanol were present in much smaller amounts, and no acetate accumulation was detected. In contrast to Gibbs' results, we did not observe a stoichiometric photoconversion of starch into H_2 and CO_2 under our experimental conditions, though we did observe a H_2:CO_2 production ration of 3.7:1 (mol:mol) . As seen in Fig. 8, very little starch was mobilized during the H_2-producing stage of the system. However, significant loss of protein takes place concomitantly with the H_2 production, suggesting that protein is the primary source of substrate and reductant for the electron transport process that eventually feeds into the reversible hydrogenase pathway. Clearly, more work is needed to accurately define the stoichiometries of the substrate catabolized, and H_2 and CO_2 photobiologically generated in this H_2 production process.

The actual rate of H_2 gas accumulation during the early times of measurement (ca. 50 h) was only 10% of the maximum capacity for H_2 evolution by the cells at this time. This discrepancy suggests that factors other than the activity of the hydrogenase might be limiting the process of H_2 gas accumulation. Some of these factors could include:

- Inhibition of the H_2-evolution process in the culture bottle due to the equilibrium constant of the reaction catalyzed by the reversible hydrogenase. Dissolved H_2 gas in the growth medium could lower the equilibrium constant of the reaction $2H^+ + 2e^- <-------> H_2$, thereby limiting the rate of the forward (H_2-evolution) reaction. In the polarographic measurements of H_2 evolution with a Clark-type electrode, this limitation does not apply since all gases in the electrode chamber were thoroughly purged by bubbling with inert argon gas.
- Losses of H_2 gas either because of improper sealing of the gas collection system or because of diffusion of H_2 from the graduate cylinder through the water phase to the surrounding atmosphere.
- Inherent competition for reductant (electrons from reduced ferredoxin) between the reversible hydrogenase, leading to H_2-gas production, and the Benson-Calvin cycle, leading to the reduction of inorganic carbon into sugar precursors.
- Differences in the effective intensity of the light used to induce H_2 evolution in the culture bottle during stress* and the light used to measure the H_2 evolution capacity of the cells in the Clark-electrode assay cell.

Clearly, additional work needs to be undertaken in order to address issues of yield and optimization of this H_2-production process. However, we have cycled a single algal culture between the two stages (oxygenic photosynthesis and H_2–production) up to three times, demonstrating the reversibility of the process (data not shown).

In summary, the ability of green algae to photoproduce H_2 gas has been a biological curiosity for many years. Up until this point, only traces of H_2 could be detected for very short periods of time using a Clark-type H_2 electrode or a mass spectrometer. The present work has shown, for the first time, that it is possible to produce and accumulate significant volumes of H_2 gas using *C. reinhardtii* in a sustainable photobiological process that can be employed continuously for several days. The process depends on a physiological treatment of the algal culture, and not mechanical or chemical manipulations. The significance of this work for future renewable energy applications remains to be examined.

Acknowledgements

The work was supported by the US DOE Hydrogen R&D Program under DOE-UC Berkeley Cooperative Agreement Number DE-FC36-98GO10278 (AM), and under contract number DE-AC36-98-GO10337 (MLG and MS). MF gratefully acknowledges support from the Swiss National Science Foundation in the form of a grant for prospective researchers. The authors wish to thank Dr. John R. Benemann for his critical reading of the manuscript and Dr. Elias Greenbaum for sharing unpublished data.

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Abbreviations: Chl, chlorophyll; Cyt, cytochrome; PSI, photosystem-I; PSII, photosystem-II; PQ, plastoquinone; P700, the reaction center chlorophyll of PSI; Q_A, the primary quinone electron acceptor of PSII; $NADP^+$, oxidized nicotinamide adenine dinucleotide phosphate; DCMU, 3'-(3,4-dichlorophenyl)-1,1-dimethylurea; TC, thermal conductance; HPLC, high-performance liquid chromatography

* For proprietary reasons, a description of the stress* condition is not disclosed.