

Reconstitution of a System for H₂ Evolution with Chloroplasts, Ferredoxin, and Hydrogenase

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Biophotolysis, H₂ Production, Hydrogenase, Ferredoxin, Chloroplasts

Continuous light-dependent H₂ production was studied in a reconstituted *in vitro* system using *Spinacea oleracea* chloroplasts, *Clostridium pasteurianum* hydrogenase and *Spirulina maxima* ferredoxin. Photosystem II-dependent production at 30 °C is 60–70 µmol H₂/mg chlorophyll. At 15 °C, this reaction proceeds for up to 20 h producing 10 µmol H₂/mg chlorophyll. O₂ (glucose, glucose oxidase) and peroxide (ethanol, catalase) traps do not extend the lifetime but enhance the rate of H₂ production. Stoichiometry of the coupled system for H₂ production in this system is 2 H₂ formed : 1 glucose consumed. A conversion efficiency of water photolysis for H₂ evolution of 70% was determined from the O₂ produced, measured as the amount of glucose consumed, during the first 2 h of continuous illumination.

Cessation of H₂ production by the reconstituted system involves inactivation of photosystem II and a limitation in the coupling of low potential electrons to hydrogenase. Increasing ferredoxin leads to more rapid H₂ evolution but longevity of the system remains unchanged. When H₂ evolution ceases due to inactivation of water-splitting activity of photosystem II, about 40% of the hydrogenase and 25% of photosystem I activity are still present; inactivation is unclear when photosystem I is used to drive H₂ production since when H₂ production ceases, hydrogenase and photosystem I still retain activity. This may suggest that coupling between low potential reducing equivalents from photosystem I to hydrogenase is impaired.

Introduction

Coupling of the reducing power of chloroplasts to *Chromatium* hydrogenase was first demonstrated by Arnon *et al.*¹ who produced H₂ using cysteine as the donor for photosystem I activity; a similar system employing dithiothreitol as the donor has been reported by Ben-Amotz and Gibbs². Photosynthetic production of H₂ in the absence of added donors, and thus presumably from H₂O, was reported by Benemann *et al.*³ using isolated spinach chloroplasts with ferredoxin and hydrogenase from *Clostridium kluyeri*. This system had an initial activity of about 1.7 µmol H₂/mg chlorophyll·h but after 15 min only 25% of the activity survived. Krampitz⁴ also reported H₂ production by coupling

isolated spinach chloroplasts to crude *Escherichia coli* hydrogenase using reduced viologen dyes to mediate electrons. Rao *et al.*⁵ improved the system by including O₂ (glucose, glucose oxidase) and H₂O₂ (ethanol, catalase) traps in order to scavenge the photosynthetically-produced O₂ (using a *Clostridium pasteurianum* hydrogenase); at 20 °C initial rates of up to 12 µmol H₂/mg chlorophyll·h were achieved, which averaged 5–6 µmol H₂/mg chlorophyll·h over a period of 3 h. With an *E. coli* homogenate as the source of hydrogenase, production of H₂ could be sustained for 6 h at a rate of 10 µmol H₂/mg chlorophyll·h.

In a preliminary report, we found that at 15 °C in the presence of BSA as a free fatty acid scavenger, that photosystem II-dependent H₂ production can be extended to up to 24 h^{6,7}. This investigation details our efforts to analyze the factors that affect the reconstitution of the coupled chloroplast-ferredoxin-hydrogenase reaction system for H₂ production.

Materials and Methods

Chloroplasts

Deveined spinach leaves were ground at 0–4 °C in MES sorbitol buffer¹ (MES·KOH 50 mM, pH 6.3; sorbitol 330 mM containing 1 mM MgCl₂), cen-

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Abbreviations: MES, 2 (N-morpholino) ethane sulphonic acid; BSA, bovine serum albumin; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPIP, 2,6-dichlorophenol indophenol; EDTA, ethylenediamine tetraacetic acid; HEPES, N-2-hydroxypiperazine-N'-2-ethanesulphonic acid; TAPS, tris(hydroxymethyl) methylaminopropane sulphonic acid.



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trifuged and a precipitate collected between 300 × *g* for 3 min, and 3000 × *g* for 10 min by resuspending in HEPES-Sorbitol buffer [(HEPES·KOH) at pH 7.0; sorbitol 330 mM containing NaCl 10 mM; MgCl₂ 1 mM; MnCl₂ 1 mM EDTA 1 mM] and kept as 1 mg chlorophyll/ml in the dark at 0 °C. O₂ evolution and uptake were measured polarographically with a Clark O₂ electrode. Chlorophyll was determined according to Arnon⁸.

Hydrogenase

Hydrogenase (EC 1.12.11) from *C. pasteurianum* (Microbiological Research Establishment, Salisbury, Proton, England) was isolated according to Nakos and Mortenson⁹, except that the bacterial pellet was dried on a rotary evaporator prior to sonication (Mortenson, personal communication) as this increased the yield and specific activity. Some preparations were the gift of Drs. K. K. Rao and D. O. Hall of the University of London King's College. Ferredoxin and ferredoxin-NADP⁺ reductase were purified from *Spirulina maxima* according to Hall *et al.*¹⁰. Protein content was estimated from 280 nm absorbance by reference to a bovine serum albumin calibration curve. The specific activity was 470 μmol H₂/mg protein·h, measured according to Chen and Mortenson¹¹ without added ferredoxin. *Spirulina* ferredoxin was determined using the extinction coefficient of spinach ferredoxin ($E_{420\text{ nm}} = 9.7\text{ mM}^{-1}\text{ cm}^{-1}$; *cf.* ref.¹²). Ferredoxin and hydrogenase were maintained anaerobically as dense stocks in liquid N₂.

Catalase (I.U.B. 1.11.1.6) from beef liver, Bovine Serum Albumin (fraction V), and chloramphenicol were obtained through Sigma Chemical Co., St. Louis, Mo. Glucose Oxidase (I.U.B. 1.1.3.4) from *A. niger* was obtained from Worthington Biochemical Corporation, Freehold, New Jersey.

H₂ evolution

H₂ evolution was performed under N₂ in sealed air-tight 7 ml vials, each containing a 2 ml sample. The composition of the reaction mixture that yielded best results is given in Table I. Vials containing all components except hydrogenase and glucose oxidase were first made anaerobic by passing a stream of N₂ over the liquid phase for several min, then glucose oxidase and hydrogenase were injected through the rubber stoppers to initiate the reaction. H₂ evolution was detected by withdrawing 50 μl aliquots from the gas phase of the vials and injecting the samples into a Varian Aerograph Model 920 provided with a thermal conductivity detector and a molecular sieve column 5A mesh 30/60. Recorder peak heights

Table I. Reaction mixture for H₂ production.

| | [μmol/ml] |
|--|-----------|
| Type II spinach chloroplasts, (as chlorophyll) ^a | 0.056 |
| <i>Spirulina</i> ferredoxin | 0.010 |
| <i>Clostridial</i> hydrogenase | 0.017 |
| Chloramphenicol | 0.250 |
| HEPES·KOH, pH 7.0 | 60.0 |
| Sorbitol | 16.5 |
| NaCl | 0.5 |
| MgCl ₂ | 0.05 |
| MnCl ₂ | 0.05 |
| EDTA | 0.05 |
| Glucose | 50.0 |
| Ethanol | 415.0 |
| | [mg/ml] |
| Bovine serum albumin | 0.5 |
| Catalase | 0.1 |
| Glucose oxidase | 0.5 |

^a Based on 500 chlorophylls per electron transport chain, this figure would correspond to 0.112 nmol of photosystem I+II units/ml.

were converted to H₂ units by reference to a calibration curve.

The reaction was performed in a specially designed shaking apparatus (Fig. 1*; W. Worthington collaboration) which holds up to eighty 7 ml rubber stoppered vials arranged in parallel rows held in a transparent plexiglass carriage. The vials are half-submerged in a temperature regulated circulating bath. Controlled linear motion of the carriage provides mixing. A bank of forty 50 W reflector lamps provides illumination through the transparent glass bottom of the water bath. Temperature regulation is assisted by blowers drawing air over the lamp bank surface. Light intensity was measured with a 12-junction Bi-Ag thermopile and a variable range voltmeter. Light intensity at the lower surface of the vials was 44–110 kergs/cm²·s. When a red polyethylene filter (cutoff $\lambda = 580$; 85% transmission above 650 nm) was interposed, the incident intensity was 23.5–90 kergs/cm²·s. Fractional intensities were obtained by means of wire mesh screens positioned between light source and sample.

The reaction was usually carried out either at 15 °C or 29–30 °C. Light-dependent H₂ evolution continues for 24 h⁶ at 15 °C. At about 26 h a light-independent production of H₂ develops. This was due to growth of a bacterium associated with spinach leaves⁶ and in long term H₂ production experiments chloramphenicol was added to prevent bacterial growth.

* Fig. 1 see Plate on page 112 a.

Glucose consumption was measured using a Worthington Biochemical Corp. Glucostat $\times 4$ Reagent Set.

Results

Requirements for H_2 productivity

A typical time course for H_2 evolution (white light, 90 kergs/cm²·s; and red light, 90 kergs/cm²·s) is shown in Fig. 2. Under N_2 at 30 °C, chloroplasts

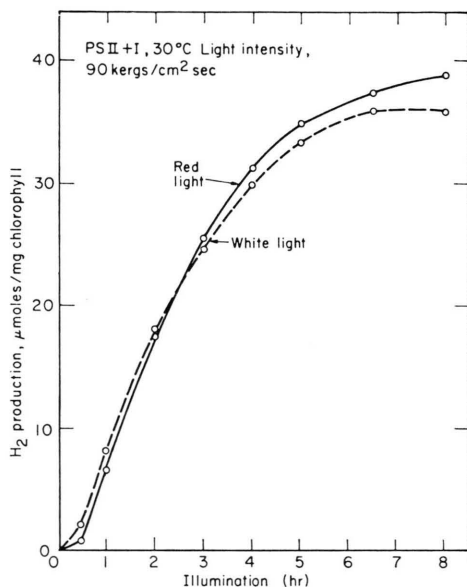


Fig. 2. H_2 evolution with continuous illumination. White light (90 kergs cm⁻² s⁻¹); red light (90 kergs cm⁻² s⁻¹) $\lambda > 580$ nm.

photolyse water to H_2 and O_2 for several h. This contrasts with the usual experience of running the Hill reaction in an aerobic medium where chloroplasts cease to photolyse water into O_2 within several minutes of continuous illumination at 25 °C. Initial rates ($\sim 11 \mu\text{mol } H_2/\text{mg chlorophyll} \cdot \text{h}$) were the same, and neither a longer functional life of the reaction system nor a higher total yield of H_2 is observed with either mode of illumination.

Maximal rate of H_2 evolution and total H_2 output are shown in Fig. 3 for various incident light intensities. Lower light intensity lowers the total H_2 yield without an appreciable prolongation of the reaction. The system saturates at light intensities that are about one-half to one-tenth of the intensities that saturate ferricyanide, or NADP⁺-supported O_2 evolution. Dark controls yield no H_2 .

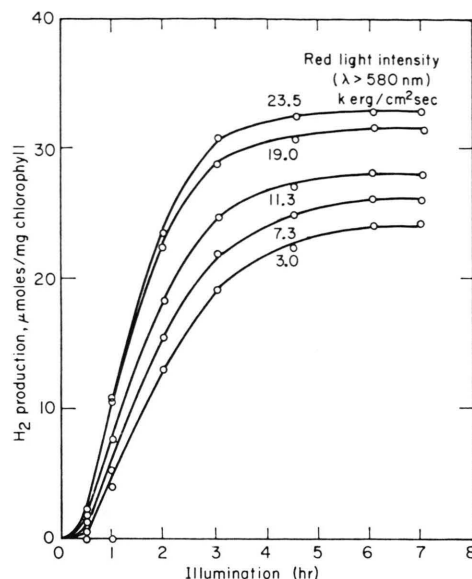


Fig. 3. Dependence of H_2 evolution upon light intensity. Numbers labeling the kinetic traces indicate intensities of incident red light illumination (>580 nm) in kergs cm⁻² s⁻¹. Dark controls were shielded from light with aluminum foil, and showed no activity.

A typical feature of H_2 evolution is the sigmoid shape of its time course. In general, we find the initial lag to be inversely related to the activity, suggesting an autocatalytic reaction. Fig. 3 shows that equal initial lags are obtained by extrapolating the kinetic curves. This is further evidence that nearly saturating light intensity was employed in the experiment. Similar initial lags were observed by Fischer *et al.*¹³ in the hydrogenase-catalyzed isotopic exchange $\text{HDO} + H_2 \rightarrow H_2O + \text{HD}$; these were satisfactorily explained by the time required for deoxygenation of the enzyme, which may explain our results.

The pH profile of the coupled H_2 production system is not only a property of hydrogenase, but also of the assay system as a whole¹⁴. In the case of a purified hydrogenase from *C. pasteurianum*, Nakos and Mortenson⁸ found an optimum pH of 7.8 for H_2 evolution mediated by reduced methylviologen, and a pH 6.8 optimum for H_2 uptake mediated by methylene blue. The pH dependence of the activity of the complete coupled system was assayed at different reaction times (Fig. 4 A). As the reaction progresses, the dependence of the yield of H_2 produced on the pH of the reaction medium becomes sharper. After 5 h illumination, a displace-

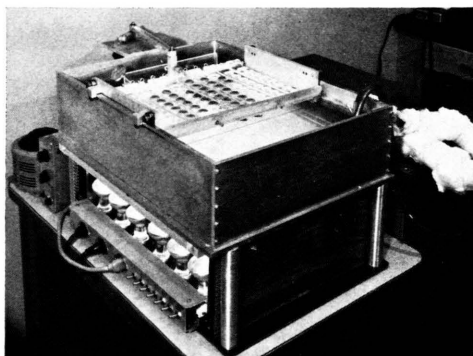


Fig. 1. H_2 evolution apparatus.

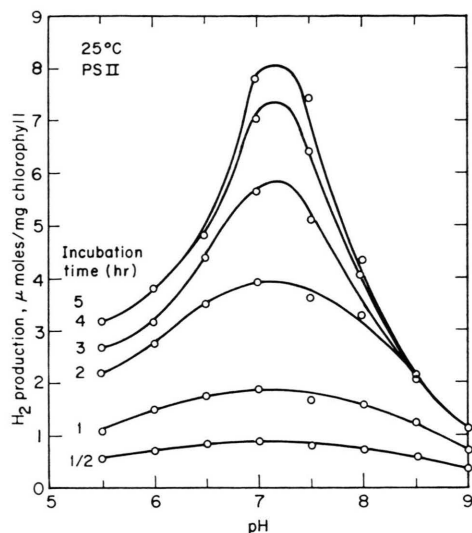


Fig. 4 A.

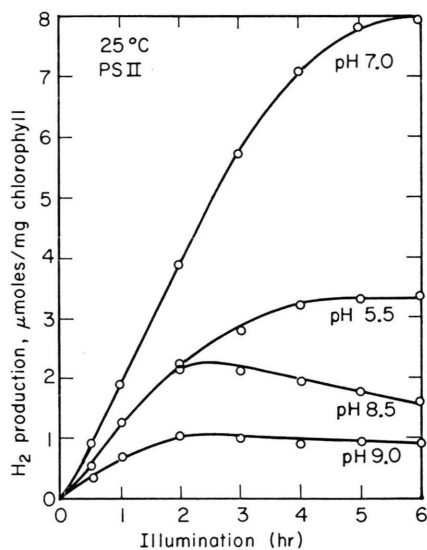


Fig. 4 B.

Fig. 4. pH dependence of H_2 production. (A) H_2 production as a function of medium pH. Numbers on the pH profiles indicate hours of reaction time elapsed. (B) Time course of H_2 evolution at various pH values of the reaction medium. Conditions as in Table I, except reaction mixtures were buffered with 100 mM of the following: TAPS plus NaOH for pH 9.0 and 8.5; HEPES plus NaOH for pH 8.0, 7.5 and 7.0; and MES plus NaOH for pH 6.5, 6.0 and 5.5.

ment by 1 pH unit on either side of the optimum (pH 7.0) results in an activity drop of about 50%. This may imply that the pH of the reaction medium has an effect on both the rate of H_2 production and

on the functional lifetime of the coupled system. Indeed, in acidic or alkaline medium, H_2 evolution ceases earlier than at the optimal neutral pH 7 (Fig. 4 B). Alkaline pH seems to be more inhibitory than acid pH, despite the well known acid lability of iron-sulphur proteins, such as hydrogenase and ferredoxin; perhaps this is because of higher aqueous H^+ concentrations.

Ferredoxin requirements in the coupled H_2 evolution system

Ferredoxin is the mediator of low potential electrons between chloroplast membranes and soluble hydrogenase. Since it is an auto-oxidizable electron carrier, it may limit H_2 output by H_2O photolysis by short circuiting electrons to reduce O_2 to O_2^- ions. In the absence of an O_2 trap, ferredoxin competes with hydrogenase and free O_2 . A second reaction limitation imposed by ferredoxin may result from its slow diffusion from the low potential electron donor (chloroplast particle) to the low potential electron acceptor (hydrogenase). Thus shorter diffusion times would be expected to enhance the rate and efficiency of H_2 production. To examine the role of ferredoxin, the kinetics of H_2 evolution was measured at different concentrations (Fig. 5 A, B). When ferredoxin concentration was increased from 1.74 to 17.42 μM , the maximal initial rate of H_2 evolution increased from 3 to 13 $\mu mol H_2/mg$ chlorophyll $\cdot h$; no H_2 is evolved in the absence of ferredoxin. Similar kinetics were observed when the reductant ascorbate/DPIP was used to supply electrons to photosystem I (Fig. 5 B) in order to bypass photosystem II.

Addition of extra ferredoxin after H_2 evolution has ceased does not revive the reaction. At that stage (normally after 6 to 8 h of continuous opera-

Table II. Analysis of residual activity after inactivation of H_2 production.

| | Dark zero time control | After illumi- nation, 8.5 h | Residual activity percent |
|--|------------------------------|-----------------------------------|---------------------------------|
| $H_2O \rightarrow$ methylviologen ^a | 756 | 0 | 0% |
| Ascorbate/DPIP \rightarrow methylviologen ^a | 492 | 122 | 25% |
| Hydrogenase ^b | 28 | 11.4 | 40% |
| Glucose oxidase ^b | ~550 | ~550 | 100% |

^a $\mu eq/mg$ Chl $\cdot h$. ^b $\mu mol H_2$ (or O_2)/mg protein/h.

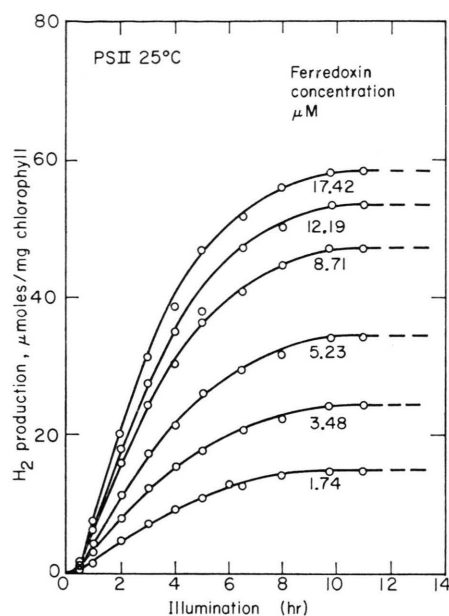


Fig. 5 A.

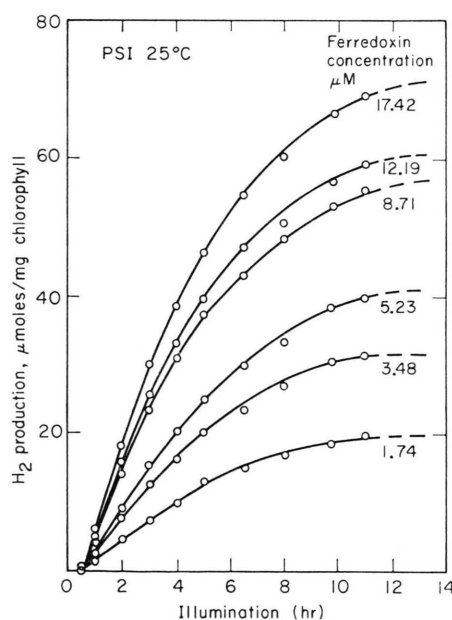


Fig. 5 B.

Fig. 5. H_2 evolution with different concentrations of *Spirulina* ferredoxin. (A) Photosystem II-driven reaction. (B) Photosystem I-driven reaction.

tion at 30 °C) about 40% of the initial hydrogenase activity and 25% of the photosystem I activity is still present (Table II). Glucose oxidase, and catalase (not shown in the table) are fully active at the

end of the H_2 evolution reaction. On the other hand photosystem II activity, as measured by methylviologen-mediated O_2 uptake, is completely lost. Addition of 7.5 mM ascorbate plus 2.5 mM hydroquinone, which presumably bypasses watersplitting activity by donating electrons directly to the photosystem II reaction center¹⁵, failed to elicit new H_2 evolution once the reaction had ceased.

In order to overcome a diffusion limitation, the possibility of substituting ferredoxin with the low molecular weight electron carrier methylviologen was investigated. Less than 2 μ mol H_2 /mg chlorophyll could be produced in the presence of 37.5 μ M methylviologen both with and without ferredoxin in the reaction mixture. Also, addition of 5 μ M phenazine methosulfate, a photosystem I cyclic electron transport catalyst, completely inhibited H_2 evolution either with H_2O or ascorbate/DPIP as the reductant.

In order to minimize the loss of reducing power by reoxidation, we explored the possibility of storing low potential reducing equivalents in a non-autoxidizable reservoir such as the NADPH/NADP⁺ couple. A prerequisite for the usefulness of this approach is a direct interaction between NADPH and *C. pasteurianum* hydrogenase yielding H_2 . We found, however, that in the absence of ferredoxin and ferredoxin-NADP⁺ reductase, NADPH alone does not couple to hydrogenase. When NADP⁺ and ferredoxin-NADP⁺ reductase is added to the basic reaction mixture of Table I, a slower H_2 evolution rate and a smaller total H_2 yield was obtained indicating a competition between hydrogenase and the pyridine nucleotides for the pool of ferredoxin electrons.

O_2 Sensitivity of H_2 production

Since the activity of photosystem II and hydrogenase are O_2 sensitive¹⁶, the role of the H_2O_2 trap in the H_2 evolution reaction mixture was examined (Fig. 6). In the absence of ethanol (but with catalase present) the activity of the coupled system is greatly reduced, and a longer initial lag of about 40 min is observed. Addition of 0.48% v/v ethanol (80 mM) stimulates the rate and the total yield of H_2 output, but has no appreciable effect on the lifespan of the system. The saturation threshold for the reaction mixture of Table I is about 2.3% v/v ethanol (400 mM). When DCMU is added to the reaction mixture, H_2 evolution ceases immediately (not shown); hence ethanol is not a photosystem I sub-

strate. When ethanol, as well as catalase, is omitted from the reaction mixture, the initial lag is shortened to 20 min. Maximal rates of H_2 evolution and total H_2 yields are approximately 2 to 5 times greater in the presence of ethanol when the trap is operating.

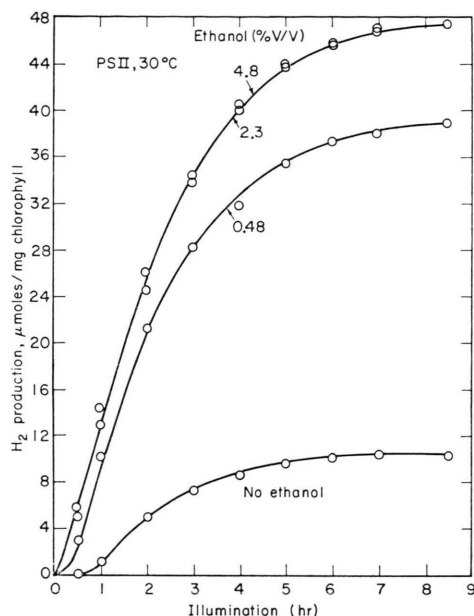
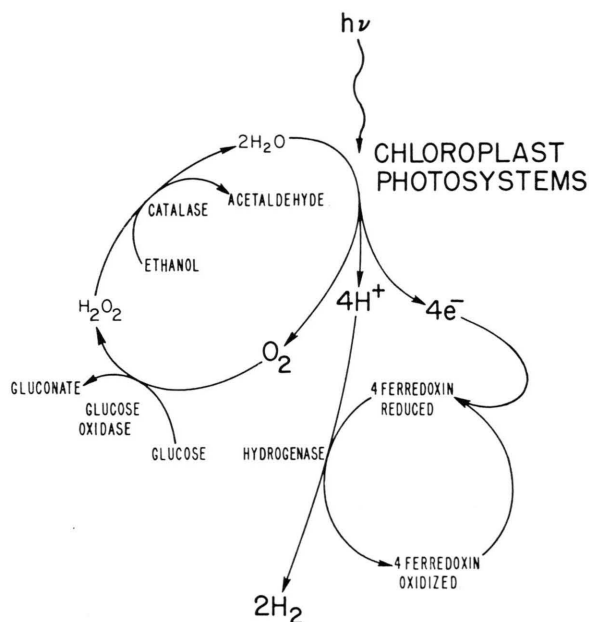


Fig. 6. Effect of ethanol on the kinetics of H_2 production.



$2H_2$ Produced: $1O_2$ Produced: 1 Glucose used

Fig. 8 A.

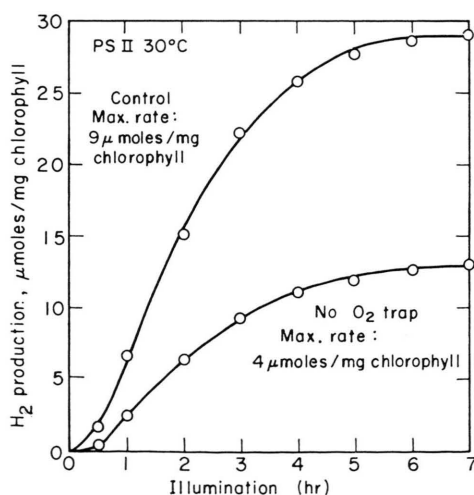


Fig. 7. H_2 evolution in the presence and absence of the O_2 and H_2O_2 traps. Conditions as in Table I except glucose and glucose oxidase and ethanol and catalase were omitted. Control and maximum H_2 rates are per hour.

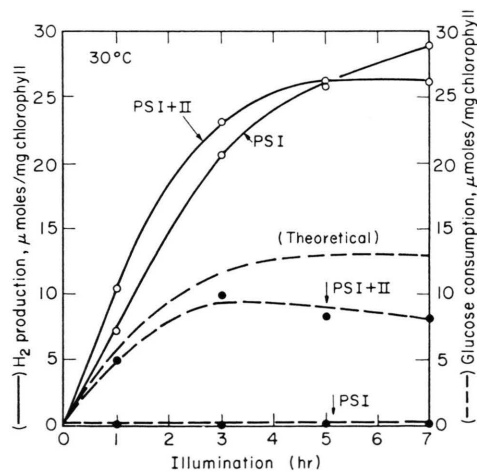


Fig. 8 B.

Fig. 8. Stoichiometry of H_2 production. (A) Scheme. (B) Photosystem II (H_2O) and Photosystem I (ascorbate/DPIP) as a source of reducing power. Glucose disappearance is used as a measure of O_2 production.

reaction system in the absence of the traps was nearly equal to that of the complete system, although both the maximal rate and the total yield of H_2 were about 65% less (Fig. 7). As noted above, further addition of ferredoxin fails to revive H_2 evolution in the inactivated reaction system.

Further experiments were carried out using the O_2 trap in order to study the efficiency of water-splitting for H_2 production by the coupled system by determining the stoichiometry of glucose disappearance : H_2 produced. Theoretically, a 1 : 2 correspondence between glucose disappearance and H_2 production should be obtained. Approximately 70% of the H_2 produced was accompanied by an equivalent disappearance of glucose (*cf.* Fig. 8 and Discussion). Thus it is unlikely that glucose or ethanol, the organic substances added to drive the O_2 and peroxide traps, are acting in any manner other than as scavengers of O_2 produced by photosystem II activity.

Discussion

Rate limiting factors of H_2 production

The overall H_2 producing process seems diffusion limited at the level of ferredoxin. Prompt cessation of H_2 evolution in the presence of phenazine methosulfate or methylviologen, compounds which effectively short circuit the low potential electrons to high potential electron acceptors, point out the difficulty of coupling photosynthetically-generated reducing power to a soluble hydrogenase. Considering the auto-oxidizability of electron carriers, *i. e.* competition by O_2 and hydrogenase for low potential electron donors, a rate limitation due to interaction of ferredoxin with chloroplast particles and soluble hydrogenase would be expected, nevertheless rather high efficiencies of conversion for the coupled reaction have been found.

Further evidence for a diffusion imposed limit on the rate of the H_2 evolution is indicated by the dependence on incident light intensity. The maximal rate of H_2 production and the maximal yield of H_2 appear to be nearly saturated at intensities as low as 8 kergs/cm²·sec, which is <10% of the light intensity required to saturate electron transport by spinach chloroplasts *in vitro*. Thus the likely explanation for the low light intensity saturation threshold is a reaction bottle neck located beyond

the stage of photoproduction of low potential reducing equivalents. At 30 °C, pH 7 and with illumination by saturating intensities of white light we observed rates of 15–20 $\mu\text{mol } H_2/\text{mg chlorophyll}\cdot\text{h}$ produced- sustainable for about 2 h, and total outputs of 60 or 70 $\mu\text{mol } H_2$ produced/mg chlorophyll for photosystem II + I and photosystem I-driven activity. These rates are <20% of the usually observed initial rates of ferricyanide or NADP⁺-supported O_2 evolution by isolated spinach chloroplasts. This could result from diffusion limitation (or lower overall electron transport rates observed above 25 °C), (S. Reeves, personal communication). The low turnover of the coupled system may be the rate limiting factor for the yield of this system, and if no alternative electron acceptor is present the rate of H_2 production probably determines the rate of water photolysis by a feedback mechanism.

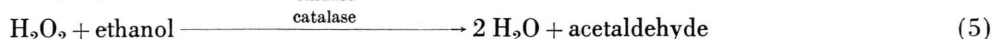
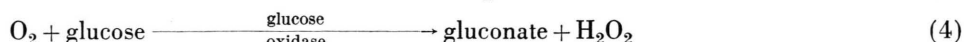
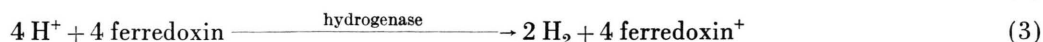
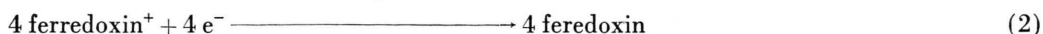
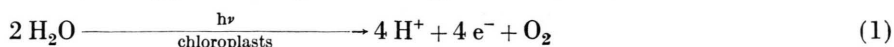
Low potential iron sulphur proteins like ferredoxin and hydrogenase vary widely as in their mid-point potentials. This may explain why methylviologen could couple isolated chloroplasts to *E. coli* hydrogenase⁴, whereas we found this was not feasible with clostridial hydrogenase. Further studies with different low potential electron carriers and hydrogenases with various oxidation-reduction potentials in the absence of side reactions are required to clarify the factors effecting the efficient interaction between these two catalysts.

Stability of H_2 production

H_2 production by a chloroplast-ferredoxin-hydrogenase system is relatively unstable, although very substantial progress has been made in maintaining this activity *in vitro*; at 30 °C H_2 evolution continues for up to 8 h with a total output of 60–70 $\mu\text{mol } H_2/\text{mg chlorophyll}$, and a maximal rate of 15–20 $\mu\text{mol } H_2/\text{mg chlorophyll}\cdot\text{h}$ which can be sustained for nearly 2 h. This activity compares favorably with earlier reports of experiments at 20 °C³ where an initial rate of <2 $\mu\text{mol } H_2/\text{mg chlorophyll}\cdot\text{h}$ recorded during the first 15 min declines to <25% of the activity within an hour.

The system is O_2 sensitive; our findings concur with Rao *et al.*⁵ who reported that H_2 production is greatly stimulated by the presence of O_2 and H_2O_2 traps with the exception that care must be taken to optimize conditions for their operation. Under these circumstances the trap works effectively and

an almost ideal 1 : 2 stoichiometry of H₂ produced: glucose disappearance (70%) was found.



2 H₂ produced : 1 O₂ produced

This is a remarkably high efficiency for coupling of the low potential reducing power of photosystem II + I to H₂ production. Contrary to our expectation, however, no apparent extension of the functional lifetime of the coupled system was noted by the presence of these traps although the rate and efficiency of H₂ production is enhanced when they are present. At the end of the reaction O₂ exceeded stoichiometrically both ferredoxin and hydrogenase by 20 times or more, yet H₂ evolution proceeded for 6 h. It appears then that O₂ produced by photosystem II activity is *per se* not the cause of the inactivation of H₂ production. Ferredoxin auto-oxidation by the photosynthetically-produced O₂ to form O₂^{•−} can be one factor that sets a limit on the lifetime of the coupled system³, and many hydrogenases lose activity on oxygenation or oxidation¹³. Ascorbate is known to exhibit superoxidase dismutase activity and its presence in unwashed chloroplasts, or as added substrates in chloroplasts utilizing photosystem I for H₂ production, may prolong the

lifetime of the coupled system. However, in other studies negligible amounts of malondialdehyde were observed in the coupled system, eliminating lipid peroxidation as a possible major inhibiting mechanism.

The ferredoxin concentration is critical for increasing the initial rate and net yield of H₂ formation. After inactivation of the reaction, it fails to elicit new H₂ production even though substantial hydrogenase and photosystem I activity remains and the efficiencies of the O₂ and H₂O₂ traps remain unchanged.

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¹ D. I. Arnon, A. Mitsui, and A. aPneque, *Science* **134**, 1425 [1961].

² A. Ben-Amotz and M. Gibbs, *Biochem. Biophys. Res. Commun.* **64**, 355–359 [1975].

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