PROLONGED PRODUCTION OF HYDROGEN GAS BY A CHLOROPLAST BIOCATALYTIC SYSTEM

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Summary The evolution of H_2 gas in an in vitro illuminated chloroplast plus hydrogenase system was shown to function for six and a half hours at a continuous rate of about 10 μmoles H_2/mg. chlorophyll/hour. Chloroplasts from various plant species, using different isolation media, and storage in a fixed state (glutaraldehyde) at 4°,-5° and-196° were shown to catalyze H_2 production. Both Clostridium and E. coli hydrogenase were used. From the use of the photosystem II inhibitors DCMU and DBMIB and heat inactivation of photosystem II, it was concluded that H_2O was the source of electrons for H_2 gas production.

INTRODUCTION Biophotolysis of water by algal and plant systems to produce hydrogen gas on a continuous basis has become a challenging problem for those involved in biological energy production. The basic aim is to use water and sunlight to generate H_2 gas in a biologically-based system (analogous to the electrolysis of water) utilizing chloroplast membranes and the enzyme hydrogenase as catalysts.

The coupling of spinach chloroplasts and partly purified Chromatium hydrogenase for photoproduction of H_2, using cysteine as the electron donor, was demonstrated by Arnon et al[1] in 1961. Then in 1973 Benemann et al[2] in a significant paper, reported the photoproduction of H_2 using water as electron donor with a chloroplast-ferredoxin-hydrogenase system. The electrons derived from the photolysis of water at photosystem II were transported to photosystem I where they reacted with the protons of the medium in the presence of ferredoxin and hydrogenase to evolve H_2 (Fig. 1). Thissystem lost 50% of its activity after 15 minutes and the authors stated that "even though the data presented indicate that O_2 is produced during the reaction, it remains to be established whether O_2 is actually evolved by the basic system".

Krampitz[3] succeeded "to produce H_2 by coupling the reducing power created

by the photolysis of water by washed spinach chloroplasts with a crude hydrogenase from the bacterium E. coli", using viologen dyes as electron carriers instead of ferredoxins. Recently Ben-Amotz and Gibbs[4] have reported light-dependent H_2 evolution with dithiothreitol, as electron donor to photosystem I, with cell free preparations of some anaerobically adapted algae and with apinach chloroplasts mixed with an algal hydrogenase.

We report here the evolution of hydrogen from an illuminated chloroplast-hydrogenase system which has functioned for up to six and a half hours. We have also simplified the requirements of the system and have determined by various control experiments that the electron transport pathway of chloroplasts was functional during the period of photoevolution of hydrogen. We also present hydrogen evolution data obtained with chloroplasts stabilized by glutaraldehyde fixation and with chloroplasts stored under various conditions.

METHODS AND MATERIALS. Chloroplasts were isolated from leaves of market spinach (Spinacia oleracea), lettuce (Latuca sativa) (Webb or Cos variety) or greenhouse grown tobacco (Nicotiana excelsior) in a sorbitol medium according to the procedure of Reeves and Hall[5] yielding type B chloroplasts. Instead of using a sorbitol-HEPES† medium, chloroplasts were also isolated using a grinding and suspension medium containing 0.2M NaCl and 50mM sodium pyrophosphate adjusted to pH 7.5, yielding type C chloroplasts. Glutaraldehyde fixation of chloroplasts was carried out by the method of West and Packer[6]. Spinach chloroplasts isolated in sorbitol medium were incubated with 0.05% (v/v) glutaraldehyde for 20 minutes at 0°C, centrifuged at 1000 g, washed and resuspended in the pH 7.6 sorbitol medium. The extent of fixation was assessed from the osmotic properties of the chloroplast measured at 546 nm.

Hydrogenase was prepared from Clostridium pasteurianum cells grown with ammonium salts as nitrogen source (obtained from the Microbiological Research Establishment, Porton, U.K.). A protamine sulphate-treated preparation of

† Abbreviations: HEPES : (N-2-Hydroxyethylpiperazine -N'-2-ethanesulphonic acid; DCMU : 3-(3,4-Dichlorophenyl)-1,1-dimethylurea ; DBMIB : 2,5-Dibromo-3-methyl-6-isopropyl-p-benzoquinone ; DCPIP 2,6-Dichlorophenol indophenol.

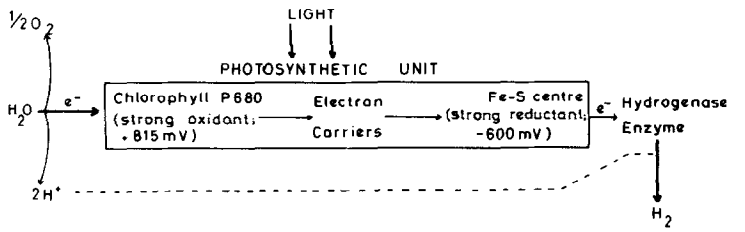

Figure 1

Coupling of solar energy to H_2 production using stabilized chloroplast membranes + hydrogenase enzymes

the cells, made according to Nakos and Mortenson[7], was chromatographed on DEAE cellulose (Whatman DE52) using a KCl gradient. Fractions eluted with 0.2M KCl were concentrated by ultrafiltration on an Amicon Diaflo PM10 membrane and the concentrated material was rechromatographed on an Ultrogel Ac 54 (LKB) column using a KCl gradient. A golden yellow fraction eluting out of the gel column contained most of the hydrogenase activity. All the operations for the isolation of hydrogenase was carried out in a N_2 atmosphere. We have also used a crude E. coli K12 strain EP 0069 (MRE, Porton) homogenate, prepared by sonication and centrifugation, as a source of hydrogenase. Spinach ferredoxin was prepared by standard procedures[8].

Hydrogen evolution was assayed in 10 or 15 ml. glass vials fitted with Suba-seal rubber stoppers. All the reactants except hydrogenase were added to the vials, sealed with the rubber stoppers and flushed with N_2 at room temperature. The vials were placed in a shaking water bath at 20°C. Hydrogenase was then injected into the vials which were illuminated by 40W incandescent lamps, giving a light intensity on the vials of about 11000 lux. At various intervals 10 to 100 µl gas samples were withdrawn with a syringe and injected into a gas chromatograph. The gas chromatograph (Taylor Servomex, Crowborough, Sussex) fitted with a Poropak Q molecular sieve column and a microkatharometer thermal conductivity detection device has a very high sensitivity for H_2 and can be used to measure H_2 in nanomolar quantities. With N_2 as the carrier gas hydrogen is easily separated from other gases - H_2, O_2, air and argon served as standards which were checked daily. Under our

operating conditions one nanomole of H_2 injected into the gas chromatograph gave a peak height of 15 cm. after a retention time of 12.5 seconds.

RESULTS AND CONCLUSIONS As seen from Fig. 2 the H_2 evolution system was functioning effectively even after 3 hr continuous illumination. The maximum lifetime we have so far observed was six and a half hours using sorbitol-isolated spinach chloroplasts and E. coli homogenate. However, chloroplasts isolated in NaCl-pyrophosphate buffer and suspended in 50mM pyrophosphate were also as effective in H_2 evolution as the basic system. Chloroplasts isolated from lettuce and tobacco leaves were also active in H_2 evolution. Bacterial or plant-type ferredoxins can substitute for spinach ferredoxin ; the exact requirement for ferredoxin was not determined as our

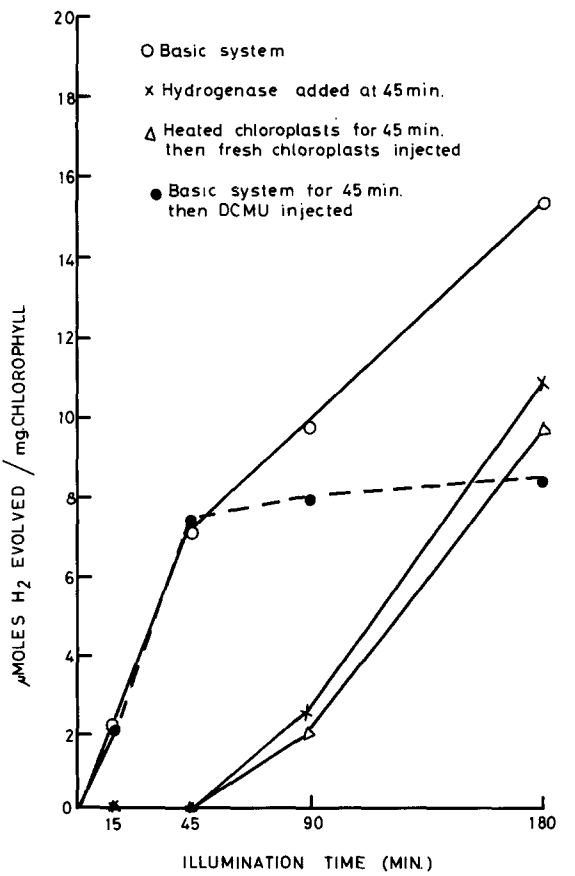

Figure 2 Time Course of H_2 evolution. The components of the basic system were as described in Table I.

Table 1. Characteristics of the chloroplast-hydrogenase H_2-evolution system

Basic reaction system contained spinach chloroplasts equivalent to 400 µg chlorophyll, hydrogenase preparation containing 400 µg protein, 20 µmoles glucose, 10 units of glucose oxidase, 20 nmoles spinach ferredoxin, 0.1M HEPES buffer pH 7.5 to a total volume of 2 ml. Temperature of reaction 20°, light intensity 11,000 lux.

Components in the system	µmoles H_2 evolved/mg chlorophyll in 3 hours
1. Basic	15.5
2. Basic in the dark	0
3. Basic minus hydrogenase	0
4. Basic minus chloroplasts	0
5 Basic using chloroplasts heated for 5 min at 55°	0
6 Basic + 10µM DCMU	0
7 Basic + 10µM DCMU + 2mM ascorbate + 25µM DCPIP	11.0
8 Basic + 5 µM DBMIB	0
9 Basic minus glucose and glucose oxidase	2.8
10 Basic with *E. coli* homogenate (no *Clostridium* hydrogenase)	9.0

crude hydrogenase preparations and chloroplasts would have contained traces of the respective ferredoxins.

The system was inhibited by oxygen either present in the assay medium or evolved during illumination ; the rates of H_2 evolution were lowered to 10% to 50% of the control rate if an oxygen trap of glucose plus glucose oxidase was omitted from the basic system. The hydrogen evolution rates and the life span of the system were enhanced when catalase and ethanol were added

Table II Activities of normal and fixed chloroplasts after 7 days storage.

Spinach chloroplasts isolated in a sorbitol medium were fixed with 0.05% glutaraldehyde and stored under various conditions. Both freshly isolated and freshly fixed chloroplasts evolved 11.5 μmoles H_2/mg chlorophyll/hr. Assay conditions as described in Table 1.

Type of chloroplasts and temperature of storage.	μmoles H_2/mg chlorophyll/hr.
1. Unfixed, 4° (cold room)	0
2. Fixed, 4°	5.2
3. Unfixed, -5° (freezer)	4.6
4. Fixed, -5°	10.0
5. Unfixed, -196° (liquid N_2)	8.4
6. Fixed, -196°	10.0

(data not shown) suggesting a possible inhibitory effect by H_2O_2 accumulating during illumination in the presence of glucose-glucose oxidase. The fact that no H_2 evolution was observed in the dark or in the absence of chloroplasts or hydrogenase (Table I) is evidence that H_2 is formed by a light driven reaction catalysed by chloroplast membranes and hydrogenase. The absence of H_2 evolution by chloroplasts treated with 10μM DCMU or 5μM DBMIB or by chloroplasts heated at 55° for 5 minutes (all of which block the O_2-evolving capacity of chloroplasts) is evidence that the electrons are donated from photosystem II i.e. by photolysis of water. Addition of ascorbate + DCPIP to these inhibited systems restores the H_2 activity showing that photosystem I is functional in these treated chloroplasts.

In control experiments run parallel to the H_2 evolution assays it was found that the illuminated chloroplasts were able to reduce $K_3Fe(CN)_6$ or NADP as long as they were able to liberate H_2. The rate of reduction of

these oxidants were the same if they were added to the chloroplasts at the beginning of illumination or after more than an hour and a half of continuous illumination thus establishing the electron transport capacity of the illuminated chloroplasts. When 2.5 mM NADP or 5mM $K_3Fe(CN)_6$ was added to a system evolving H_2 there was an immediate inhibition of H_2 evolution ;this inhibition was temporary with NADP but irreversible with $K_3Fe(CN)_6$.

One of the problems in any type of work with isolated chloroplasts is their instability at ambient and 4°C temperatures. As seen from Table 2 fixation with glutaraldehyde and storage at -5°C maintains the H_2 evolution activity of the chloroplasts for weeks. It is also possible to preserve isolated chloroplasts at liquid N_2 temperatures for months or more.

The assays were done at 20° with a white light of intensity 11,000 lux, but the H_2 evolution rates were much higher at 30°. However, the lower temperature and light intensity were chosen to demonstrate that the process can be operated at ambient temperatures at relatively low light (about $^1/_{10}$ full sunlight).

In conclusion, our study shows that it is feasible to produce H_2 from water and sunlight for a prolonged period by a biocatalytic process using chloroplast membranes and bacterial hydrogenase. The system still has a number of limitations and a lot more research has to be performed in order to stabilize the catalysts and optimize the reaction conditions to achieve efficient production of hydrogen by the biophotolysis of water.

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