CO-IMMOBILIZATION EFFECT ON \( \text{H}_2 \) PRODUCTION 
BY 
A CHLOROPLAST MEMBRANES-HYDROGENASE SYSTEM 

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SUMMARY 
Chloroplast membranes immobilized within a BSA-GA matrix or within 
an alginate gel have been associated with native or immobilized hydrogenase in order to produce hydrogen gas through biophotolysis of water. Due to the reaction geometry, co-immobilization of chloroplast membranes with the enzyme inside the same matrix considerably improved the amount of \( \text{H}_2 \) produced and the initial activity. The use of entrapment methods such as alginate gel allowed diffusion of proteins through the matrix. Electron microscopic observations illustrated these results.

INTRODUCTION 
Hydrogen production through bioconversion of solar energy has drawn new attention since the possibility of using immobilized biosystems. It was previously reported that combined systems consisting of higher plant chloroplast membranes and bacterial hydrogenase can liberate hydrogen gas resulting from the photolysis of water (Benemann et al., 1973). Since this work progress has been made to improve the efficiency of the system (Fry et al., 1979). However, the interest or the system is severely limited both by the rapid decay of photosynthetic activity and by the necessity of removing the oxygen produced during the reaction. 

Immobilization of biocatalysts should allow the stabilization of their catalytic activity and moreover, the resulting insoluble particles might be used in continuous flow reactors (Legoy et al., 1982). 

Recently hydrogenase from \textit{Desulfovibrio gigas} has been immobilized by covalent coupling onto a porous silica support (Hatchikian and Monsan, 1980). This highly active immobilized hydrogenase shows a very good resistance to oxygen inactivation and seems suitable for hydrogen production when coupled with illuminated chloroplasts. 

Chloroplast membranes have been immobilized with retention of photosynthetic electron transport activity and improvement of their stability. Several methods have been tried such as entrapment inside an alginate gel (Gisby and Hall, 1980), and agar gel (Karube et al., 1980),
with polyvinyl alcohols (Ochiai et al. 1978) or with vinylmonomers by radiation induced polymerization (Fujimura et al. 1981), or by co-crosslinking with glutaraldehyde in the presence of albumin (Cocquempot et al. 1979). Some of these methods were recently compared (Cocquempot et al. 1981) and among them, two have been selected to study hydrogen production of reconstituted systems comprising immobilized chloroplast membranes and immobilized hydrogenase. The efficiency of the systems consisting of the two biocatalysts either immobilized on separate supports or co-immobilized inside the same support is reported.

MATERIALS AND METHODS

- Hydrogenase from Desulfovibrio gigas has been purified according to Hatchikian et al. (1978). The preparation used in the coupling assays exhibited a specific activity of 180 μmoles H₂/min/mg protein.

- Cytochrome C₃ (Cyt C₃) and Flavodoxin (Fld) from D. gigas used as mediators in the reaction have been purified as previously reported (Le Gall et al. 1965, Le Gall and Hatchikian, 1967).

- Hydrogenase has been immobilized by covalent coupling onto a porous silica support : Spherosil beads (Rhone-Poulenc Ind.) with aromatic amino groups, activated by diazotation (Hatchikian and Monsan, 1980), or onto Sepharose 4B beads supplied as Br-CN activated material by Pharmacia Fine Chemicals.

- Chloroplast membranes were isolated from market spinach as previously described (Cocquempot et al. 1979). Two immobilization methods have been followed : - a cocrosslinking method using glutaraldehyde (GA) at sub-zero temperature and in the presence of an inert protein such as Bovine-serum-albumine (BSA) (Cocquempot et al. 1979).

- an entrapment method using a calcium alginate gel previously described for cell entrapment (Ohlson et al. 1979).

- Crosslinked albumin polymer contains in each case the oxygen scavenger enzymes (glucose oxidase + catalase) and thylakoids. Spherosil or Sepharose beads bearing hydrogenase molecules were added in each co-immobilized system before the obtention of the insoluble form.

- The chloroplast photoreducing activity was tested amperometrically using methylviologen as electron acceptor as previously reported (Cocquempot et al. 1979).

- Hydrogenase activity was determined by manometric measurement of H₂ evolution using an excess sodium dithionite (15 mM) as electron donor and methylviologen (1 mM) as electron mediator.

- Hydrogen production was carried out according to the method described by Fry et al. (1977) and using glucose oxidase, glucose, catalase and ethanol as O₂ and H₂O₂ trap. Anaerobicity was obtained with flushing under argon. Each Vial was thermostated at 25°C and the reaction was started with a light intensity of 15000 Lux. H₂ evolution was estimated by gas chromatography.