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Comparative Stabilization of Biological Photosystems by Several Immobilization Procedures

2. Storage and Functional Stability of Immobilized Thylakoids

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Summary. Lettuce thylakoids were immobilized by various methods selected to provide the chloroplast membrane with different environments. These included proteins (albumin and gelatin), polysaccharides (carrageenan and alginate gels) and synthetic polymers (photocrosslinkable resins and polyurethans). Large variations were observed in the activity yield after immobilization (ranging from 3% to 70%), in the storage stability (at 4 °C in absence of light) and in the functional stability (continuous work at 20 °C under illumination).

Introduction

Essentially, photosynthesis consists of the collection of solar radiant energy and its transformation into redox and, finally, chemical energy. Chloroplast membranes (thylakoids) are able to perform the photolysis of water but lack hydrogenase activity. On the other hand, photosynthetic bacteria exhibit hydrogenase activity but are unable to perform the photolysis of water. The coupling of the photosynthetic electron flow of isolated thylakoids to bacterial hydrogenase gives rise to hydrogen production (Arnon et al. 1961; Benemann et al. 1973; Benemann 1980).

However the productivity of the system is severely limited both by the stability of the thylakoid membranes and the sensitivity of hydrogenases to oxygen (Rao and Hall 1979). In this paper we have directed our attention to the first problem by immobilizing lettuce thylakoids within various supports and measuring their subsequent stability and integrity. Several laboratories, including ours, have tried to stabilize in vitro the photochemical activity of thylakoids by microencapsulation (Kitajima and Butler 1976), entrapment within polyacrylamide gels (Ochiai et al. 1977; Karube et al. 1979) or within polyvinylalcohol polymers (Ochiai et al. 1978), adsorption onto diethylaminoethylcellulose (Shioi and Sasa 1979), crosslinking with glutaraldehyde in the presence of albumin (Cocquempot et al. 1979, 1980). Recently, the action of glutaraldehyde on the structure and function of chloroplast membranes has been the subject of an excellent review (Papageorgiou 1979).

Materials and Methods

Preparation of the Biological Material

Chloroplast membranes were isolated by blending lettuce leaves (Latuca sativa) suspended in an homogeneization medium containing 330 m*M* sorbitol, 10 m*M* sodium pyrophosphate, 20 m*M* NaCl, 1 m*M* $MgCl_2$, 1 m*M* $MnCl_2$, 2 m*M* EDTA and adjusted to pH 6.8.

Thylakoids were obtained by osmotic shock according to the method of Epel and Neuman (1973). After centrifugation at 3,000 g for 30 s, the membranes were resuspended in 50 m*M* HEPES buffer pH 7.6 containing 330 m*M* sorbitol.

Immobilization Methods

Crosslinked albumin polymer: The immobilization process is based on a method previously used for immobilizing enzymes (Broun et al. 1973) or bacteria (Petre et al. 1978). A solution was produced by mixing 1.65 ml of 0.02 M pH 7 phosphate buffer, 1.25 ml of a 20% bovine serum albumin solution, 0.6 ml of thylakoids suspension corresponding to 2 mg of chlorophyll and 1 ml of glutaraldehyde solution at 1.5%. (The resulting glutaraldehyde concentration is then 0.33%.) This mixture was frozen at −20 °C during 2 h and then slowly thawed at 4 °C and rinsed.

Crosslinked gelatin polymer: A similar procedure was used with a mixture containing 1.15 ml of 0.02 M pH 7 phosphate buffer solution, 2 ml of a 10% gelatin solution, 0.6 ml of thylakoids suspension (2 mg of chlorophyll) and 0.25 ml of glutaraldehyde solution at 1%. (The resulting glutaraldehyde concentration is then 0.0625%.)

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Table 1. Activity of native and immobilized thylakoids measured with potassium ferricyanide as electron acceptor in the absence (column A) and presence of NH_4Cl (Column B)

	A μmol O_2 mg Chl^{-1} h^{-1}	B +NH_4Cl μmol O_2 mg Chl^{-1} h^{-1}
Native thylakoids	50.90	112
Crosslinked albumin polymer	67.5	76.2
Polyurethane-BSA	37.8	56
Crosslinked gelatin polymer	48.9	50.4
Urethan polymer	24.1	31.36
Alginate gels	24.7	24.7
Photocrosslinkable resins	19.4	21.3
Carrageenan gels	3.4	3.4

Alginate Gels. Calcium alginate gels have been widely used for the immobilization of enzymes, organelles or cells (Hackel et al. 1975; Kierstan and Bucke 1977; Ohlson et al. 1979).

The thylakoid suspension (0.5 ml containing 1.6 mg of chlorophyll) was mixed with 9 ml of a 2% sodium alginate solution and added to 0.05 M $CaCl_2$ as droplets with the aid of an hypodermic syringe.

Carrageenan Gels. The thylakoid suspension was immobilized inside carrageenan gels according to the procedure of Takata et al. (1979), the only modification being in the use of iota-carrageenan (which gives more transparent gels) in place of kappa-carrageenan. (Alginate and carrageenan were kindly donated by "Centre de Recherches CECA-F 50500 Carentan- Baupte").

Photocrosslinkable Resins. Standard immobilization procedure was to mix 1.5 g of a photocrosslinkable polymer (65% ENT 4000 aqueous solution) with 0.1 ml of 10% benzoïn isobutylether (initiator) in polypropylene glycol and 1 ml of thylakoid suspension. The mixture was layered on a sheet of transparent polyester and illuminated for 3 min (Fukui et al. 1976; Tanaka et al. 1978).

Urethan Polymer. The thylakoïd suspension was immobilized by mixing with urethan prepolymer (PU 3) according to the procedure of Fukushima et al. (1978). Urethan and photocrosslinkable prepolymers were kindly donated by Professor S. Fukui (Kyoto University, Japan). In some cases (mentioned as "polyurethan-BSA method") Bovine serum albumin was added to the mixture at a final concentration of 5% (V/V).

Oxygen Production

Oxygen evolution was measured amperometrically with a Clark-type electrode Ferricyanide (5 m*M*) was used as the electron *acceptor* and ammonium chloride (5 m*M*) as the uncoupling reagent (Cocquempot et al. 1979). Reaction media were illuminated at a saturating intensity (30,000 lux) by a 100 W iode lamp equipped with a focusing device and a red filter. The temperature was maintened at 20 °C and the activity of the thylakoids (either free or immobilized) is expressed as μmol of O_2 mg^{-1} of chlorophyll h^{-1}.

Electron Microscopy. The specimens were fixed and dehydrated according to the method of Barbotin and Thomasset (1980).

For transmission electron microscopy the specimens were embedded in Epon, sectionned and double stained. The micrographs were obtained using a JEOL 100 C under 80 kV.

Experimental Results

Activity Yields

The comparative oxygen production (biophotolysis of water) of immobilized and native thylakoids was measured using potassium ferricyanide as electron acceptor. The results are summarized in Table 1. As the uncoupling effect of each procedure varies (Cocquempot et al. 1979) it is necessary to add an uncoupler (NH_4Cl, 5 m*M*) to the reaction medium in order to obtain a well defined activity yield, expressing the integrity of the electron transfer chain (Column B). In the absence of ammonium chloride (Column A), the kinetic results represent the sum of two phenomena: the partial denaturation and the partial uncoupling of the electron transfer chain after immobilization. This will produce a decrease (denaturation) and an acceleration (uncoupling), respectively, of the oxygen production rate when compared with the native thylakoids. Therefore in all subsequent experiments, NH_4Cl (5 m*M*) was systematically added as uncoupling agent.

Storage Stability

Native and immobilized thylakoids were stored in the dark at 4 °C, periodically sampled and their activity was assayed as previously described. The results (Fig. 1) show the initially available activity (immobilization yield) and its evolution as a function of time. After 400 h of storage (data not shown) only polyurethan, polyurethan-BSA and crosslinked albumin polymers kept a residual activity (5, 10.5, and 17 μmol O_2 mg chlorophyll^{-1} h^{-1}) respectively.

Functional Stability

Oxygen production by native and immobilized thylakoids was continuously monitored at 20 °C under illumination (Fig. 2). After about 50 min, the native thylakoids are completely inactivated while the immobilized thylakoids still keep some residual activity. If we compare these results with those of storage stability in the dark, it becomes obvious that the continuous use under illumination accelerates the inactivation rate by a factor of about 300. Moreover, the immobilization by the BSA-glutaraldehyde procedure seems to protect the thylakoids against the

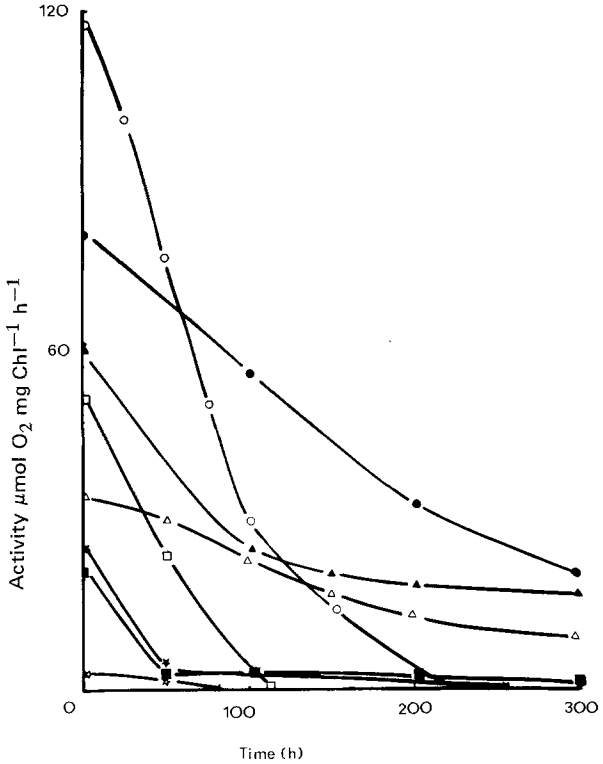


Fig. 1. Oxygen evolution measured as a function of time in storage in the dark at 4 °C for native thylakoids (–○–) and thylakoids immobilized with crosslinked albumin polymer (–●–), with crosslinked gelatin polymer (–□–) into a polyurethane matrix (-△–△-), into a polyurethane-BSA matrix (-▲–▲-), into carrageenan gel (–☆–☆–), into algniate gel (-★–★-) and into photo-crosslinkable resin (–■-■–)

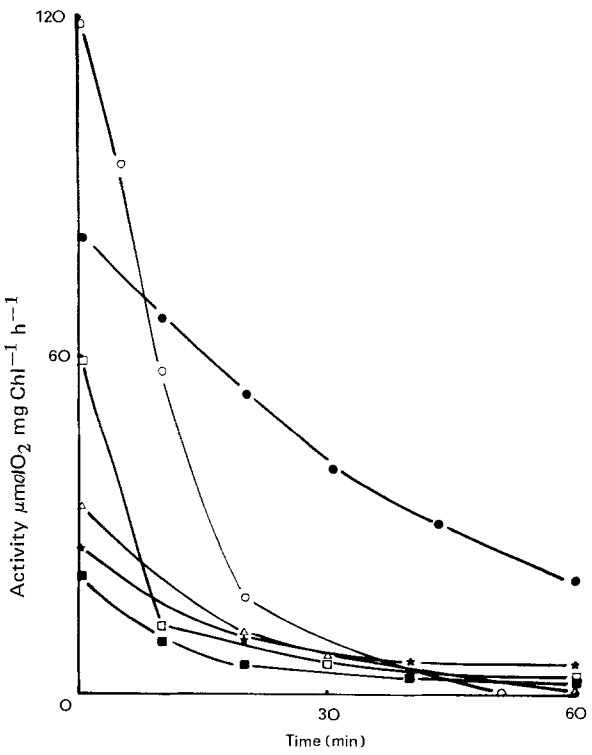


Fig. 2. Oxygen evolution measured as a function of time under continuous use with saturating illumination of native thylakoids (–○-○–) and thylakoids immobilized with crosslinked albumin polymer (–●-●–), with crosslinked gelatin polymer (–□-□–), into a polyurethane matrix (-△–△–), into alginate gel (–★–★–) and into photocrosslinkable resin (–■-■–)

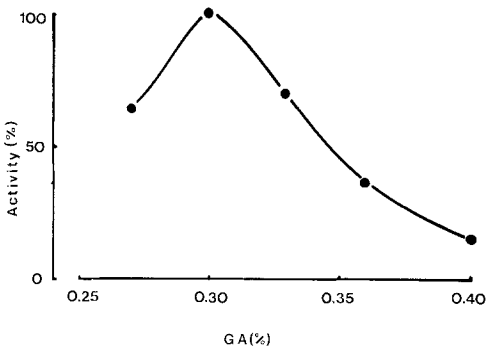
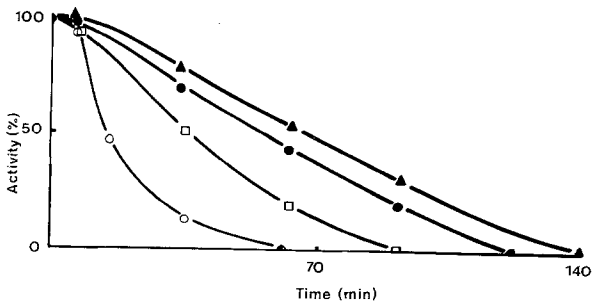


Fig. 3. Activity of thylakoids immobilized with crosslinked albumin polymer as a function of glutaraldehyde concentration. (The 100% value corresponds to 153 µmol O_2 mg chlorophyl^{-1} h^{-1})

photoinactivation to a considerable degree (see for instance Fig. 2, the comparatively poor protection provided by the gelatin-glutaraldehyde procedure).

Optimization of Thylakoid Immobilization by Albumin Glutaraldehyde Method

The standard immobilization procedure was used, except that the *glutaraldehyde concentration* (GA) was varied from 0.27% to 0.40% (V/V). The activity yield is very dependent of the glutaraldehyde concentration (Fig. 3) and for the optimal concentration (0.3%) the thylakoid



◀ Fig. 4. Activity of native thylakoids and thylakoids immobilized with crosslinked albumin polymers as a function of time under continuous use with saturating illumination. Several glutaraldehyde concentrations were used. The 100% values correspond to:
- 180 µmol O_2 mg^{-1} h^{-1} for native thylakoids (-○-○-)
- 160 µmol O_2 mg^{-1} h^{-1} for thylakoids immobilized with 0.3% of glutaraldehyde (-□-□–)
- 122 µmol O_2 mg^{-1} h^{-1} for thylakoids with 0.33% of glutaraldehyde (–●-●–)
- 80 µmol O_2 mg^{-1} h^{-1} for thylakoids immobilized with 0.35% of glutaraldehyde (–△–△)

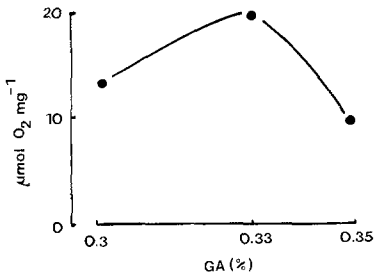


Fig. 5. Total O_2 production by thylakoids immobilized with crosslinked albumin polymer as a function of glutaraldehyde concentration during continuous use under illumination

activity is recovered almost quantitatively (85% and 90% respectively for duplicate results). However the functional stability (continuous use under illumination) of the immobilized thylakoids was further improved when using higher glutaraledhyde concentrations (Fig. 4), although of course at the cost of a decreasing activity yield. In terms of productivity (total oxygen production) the optimal value for the glutaraldehyde concentration (Fig. 5) was found to be 0.33% (V/V) representing a compromise between activity yield (decreasing with increasing GA concentrations) and the stabilization (increasing with increasing GA concentrations).

Electron Microscopy

Ultrathin sections of crosslinked albumin polymers bearing immobilized thylakoids reveal a greater degree of stacking of the grana array (Fig. 6b) than observed with the suspension (Fig. 6a). Such appearance can explain the increase of stability of the immobilized thylakoïds. On the other hand the albumin matrix exhibits an homogeneous internal structure without pores (Fig. 6b).

The urethan-BSA polymer section (Fig. 6c) indicates a less dense structure than in the above case. In this example, the immobilized thylakoïds retain their structural integrity.

The Fig. 6d shows an ultrathin section of calcium alginate polymer. A sponge-like structure composed of

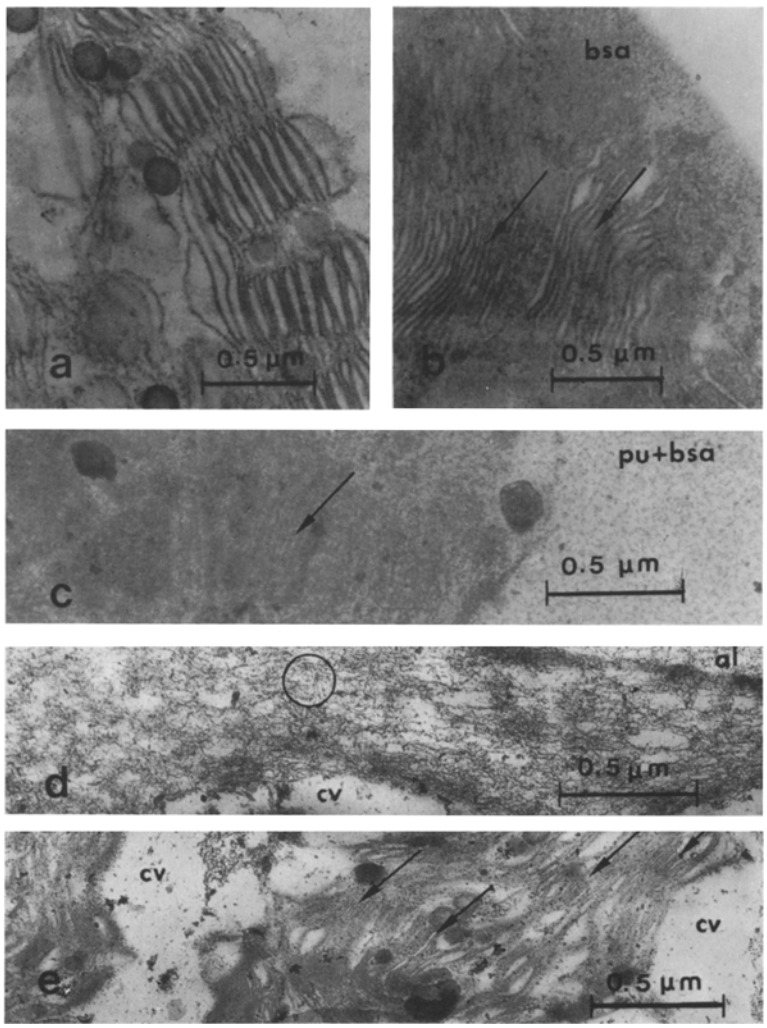


Fig. 6a–e
Electron Microscopy: Ultrathin sections
a Lettuce thylakoid showing an array of membranes
b Immobilized thylakoid into albumin foam structure. The *arrows* indicate an extensive array of parallel membranes. Note the homogeneity of the albumin structure
c Immobilized thylakoïd into polyurethane albumin structure. The *arrow* indicates the presence of membranes
d Alginate structure. Cavities (*cv*) can be distinguished. The *circle* shows the assembly of thin filaments in the matrix frame
e Immobilized thylakoid into alginate structure. Note the presence of numerous cavaties (*cv*) around the thylakoïd

thin filaments and numerous cavities can easily be distinguished. The entrapped thylakoids (Fig. 6e) appear, inside cavities, with an irregular stacking aspect.

Discussion

The biophotolysis of water is attracting considerable attention in view of its long-term potentialities for the production of storable, non-polluting and energy-rich molecules (oxygen and hydrogen) from unlimited energy (sun) and substrate (water) sources. Since the report of Benemann et al. (1973) the hydrogen productivity (calculated per mg of chlorophyll) of the chloroplast-hydrogenase system has been increased about 200 fold (Rao et al. 1979). However, there are still many problems to be solved before the system could be applied practically on a large scale. Among these limitations, the storage stability and the photoinactivation of the chloroplast membrane are obviously crucial ones. In previous papers (Cocquempot et al. 1979, 1980) we presented some results obtained with thylakoids immobilized by the action of glutaraldehyde in the presence of albumin at subzero temperature. The storage and functional stabilities of the thylakoids: were improved after immobilization. But it was difficult to assess which of the following parameters could be critical for the stability of the immobilized thylakoids:

- the temperature,
- the microenvironment,
- the formation of covalent linkages,
- the specific protective effect due to bovine serum albumin.

Therefore we considered the comparative immobilization of the chloroplast membrane within various matrices (proteins, polysaccharides and synthetic polymers) and by different crosslinking procedures (ionic or covalent) as a method to obtain more information on the above mentioned parameters. In addition, it was an opportunity to test the suitability of each of these methods.

Temperature

With the exception of carrageenan gels, all manipulations were performed at low temperature (4 °C). The gelling temperatures of a carrageenan sol being 30 to 35 °C, (Pedersen 1974), the thylakoid suspension had to be mixed at 40° to 45 °C, although only for a few minutes. Yamashita and Butler (1968) have shown that even a mild heat treatment (3 min at 50 °C) is sufficient to almost completely inactivate the elctron treansfer between water and photosystem II. This inhibition can be relieved by addition of artificial electron donors like semicarbazide, which are able to bypass the site of inhibition and to donate electrons directly to photosystem II (Yamashita and Butler 1969). Addition of semicarbazide (3 m*M*) to the reaction mixture (data not shown) restored almost quantitatively the electron flow up to ferricyanide for the carrageenan-entrapped thylakoids as compared with the native ones. These results suggest that both photosystems I and II are still active after immobilization and that the low activity yield (3%) for the oxygen production could be ascribed to the selective inactivation of the water splitting enzyme. It is also interesting to note that glutaraldehyde fixation protects the thylakoids against this selective heat inactivation (Zilinskas and Govindjee 1976).

Covalent Linkages and Microenvironment

In a separate paper dealing with bacterial chromatophores immobilized by various methods, an important increase of stability was observed whatever the matrix (Larreta garde et al. 1981). In the case of the immobilized thylakoids the results seem to be less simple. Gelatin crosslinked and carrageenan-entrapped thylakoids are even less stable than native thylakoids during storage (Fig. 1), although some protection against photoinactivation is observed (Fig. 2). Concerning the possible role of covalent bonds, the optimization of the BSA-glutaraldehyde method shows that they may have both a deleterious (activity yield) and stabilizing effect. It has been reported that the crosslinking of the chloroplast membrane by glutaraldehyde alone improves the survival of electron transport activity during dark storage but not during continuous illumination (Papageorgiou and Isaakidou 1977). Our results (Figs. 1 and 2) indicate that in presence of a high concentration of bovine serum albumin, the stabilization is observed in both cases.

Role of Bovine Serum Albumin

Bovine serum albumin was shown to stimulate various photoreactions in isolated bean and lettuce chloroplasts (Friedlander and Neumann 1968; Kahn 1966) and to be very effective in preserving the activity of isolated chloroplasts stored in vitro (Wassermann and Fleisher 1968). For a maximal protective effect, the albumin molecules have to be present *during* the homogeneization step and *continuously* in the chloroplast preparation. Similar results concerning the action of bovine serum albumin on aged rat liver mitochondria have also been reported (Helinski and Cooper 1960). These authors showed that for various albumin preparations (native, coagulated, heat denatured, immunologically active low molecular weight fragments), their ability to reverse the effects of aging corresponded with their immunological activity and affinity for long chain fatty acid anions (oleate). Unsaturated fatty acids (specially C_{18}, i.e., oleic, linoleic and linolenic acids) released during isolation, or during chloroplast

aging in vitro, or also exogenously added were shown to inhibit electron flow (Wintermans et al. 1969; Siegenthaler 1972). An addition of bovine serum albumin largely prevented the decrease of the photo-phosphorylation and of the electron transfer chain. The large differences observed with thylakoids immobilized in presence of gelatin and albumin (Fig. 2) could therefore probably be ascribed to the binding affinity of the albumin molecules for the uncoupling fatty acids released during the ageing or the photoinactivation. Whether or not the crosslinked albumin polymer still has all its binding site available remains to be proven. Moreover, as the bioconversion of hydrophobic compounds is gaining an increasing interest, we are now investigating the properties of these proteic polymers in terms of affinity constants and partition coefficients towards non-polar molecules.

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