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Immobilization of a Photosystem II Submembrane Fraction in a Glutaraldehyde Cross-Linked Matrix

ROBERT CARPENTIER* AND SYLVIE LEMIEUX

Centre de Recherche en Photobiophysique, Université du Québec à Trois-Rivières, C.P. 500, Trois-Rivières, Québec, G9A 5H7, Canada

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ABSTRACT

A wide range of immobilization procedures have been shown to stabilize the functions of photosynthetic materials. The purpose of this work was to determine if the above procedures can be applied to submembrane fractions. Triton X-100-derived photosystem II submembrane fractions isolated from spinach were immobilized in a glutaraldehyde cross-linked albumin matrix. The optimal conditions were obtained in presence of 1 m*M* NaCl and 5 m*M* $MgCl_2$. The treated membranes were less affected by long-term storage at 4°C, high pH and temperature, and strong light exposure. The results are discussed in terms of a diffusion barrier resulting from the immobilization matrix.

Index Entries: Submembrane fractions immobilization; glutaraldehyde effect; photosystem II.

INTRODUCTION

The capture of solar energy by the pigmented photosynthetic membrane leads to charge separation, with the resultant production of negatively charged species and water photolysis. These properties have been used in electrochemical cells containing not only chloroplast or thylakoid membranes (*1–4*), but also photosystem I particles and bacterial reaction centers (*5–10*). However, the active lifetime of isolated chloroplast mate-

*Author to whom all correspondence and reprint requests should be addressed.

rial is too short for applications of biotechnological importance, and prior immobilization is necessary. Various immobilization techniques have been developed in order to improve the stability of biological functions. For example, isolated chloroplasts or thylakoids were immobilized by microencapsulation within a permeable membrane (*11*), by adsorption onto cellulose derivatives (*12*), by gel entrapment (*13–15*), by protein crosslinking or polymerization (*16,17*), or through cocross-linking in a protein matrix (*18*). The improved thylakoid stability under temperature stress, long-term storage, and continuous illumination after immobilization was correlated with a greater functional stability for photochemical hydrogen evolution or photocurrent generation (*19–21*).

The previous successful use of a submembrane fraction in photoelectrochemical cells (*5–8*) and the increasing interest in isolated photosystems for the study of specific photoreactions raised the question about the applicability of the immobilization procedures to submembrane fractions. The immobilization of thylakoid membranes in a glutaraldehyde–albumin cross-linked matrix was shown to give better stability when compared to several other techniques (*22,23*). In this report, we describe the application of the above immobilization procedure to an oxygen-evolving photosystem II submembrane fraction. The use of such preparation was motivated by its ability to provide both charge separation and oxygen evolution. We have studied the tolerance of the immobilized photosystem II to pH, temperature, and long-term storage at 4°C. Their resistance to saturating light exposure was also analyzed in comparison with the native material.

METHODS

Photosystem II submembrane fractions were isolated from spinach according to Berthold et al. (*24*), with the exception that the buffers were maintained at pH 6.5 instead of pH 6.0. The resultant preparation was kept in 20 m*M* Mes–NaOH, pH 6.5, with a Chl concentration of 2 mg/mL and stored at 4°C. Salts (NaCl and/or $MgCl_2$) were added at concentrations specified in the text. Chlorophyll was determined according to Arnon (*25*).

The photosystem II particles were immobilized following a slight modification of the procedure described by Thomasset et al. (*18*). Photosystem II (0.6 mL) was mixed with 1.65 mL of 20 m*M* Mes–NaOH, pH 6.5, 1.25 mL of 20% bovine serum albumin, and 1 mL of 1.5% glutaraldehyde. After mixing, the solution was divided into 75-μL samples and frozen at −20°C for 2 h. The samples were thawed for 2 h at 4°C and homogenized in a mortar containing the appropriate buffer before use.

Initial rates of oxygen evolution were monitored with a water jacket Clark type of electrode at 22°C, as described elsewhere (*26*). The reaction medium contained 11 μg Chl/mL, 600 μ*M* 2,6-dichlorobenzoquinone, and 20 m*M* Mes–NaOH, pH 6.5. Additional salts were added to the reac-

tion medium as specified in the text. When oxygen evolution was determined as a function of temperature, the samples were incubated in the oxygen electrode cell thermostated at the appropriate temperature for 5 min before measurement. In the pH studies, 20 m*M* Mes–NaOH, 1 m*M* NaCl, and 5 m*M* $MgCl_2$ were used as the medium for pH's between 5 and 6.5; and 20 m*M* tricine–NaOH, 1 m*M* NaCl, and 5 m*M* $MgCl_2$ were used for pH's between 7.0 and pH. 8.0. The samples were equilibrated 2 min in the proper medium before measurements.

Pigment photobleaching was measured at 680 nm according to Carpentier et al. (*27*) in 20 m*M* Mes–NaOH, 20% Ficoll, 1 m*M* NaCl, 5 m*M* $MgCl_2$, and 11 μg Chl/mL. The photoinhibition of electron transfer was performed in the oxygen electrode cell. The sample was illuminated (white light, 2500 $\mu E/m^2/s$) for a given time under aerobic conditions in 20 m*M* Mes–NaOH, 1 m*M* NaCl, and 5 m*M* $MgCl_2$. Then, 600 μ*M* 2,6-dichlorobenzoquinone was added in the dark and oxygen evolution was monitored as above. The rates were corrected with a dark control.

RESULTS

We applied the procedure described by Thomasset et al. (*18*) to immobilize a Triton X-100-derived photosystem II submembrane fraction. The methodology described in ref. (*18*) implied the mixture of glutaraldehyde, albumin bovine, and chlorophyll-containing membranes in a phosphate buffer, pH 7.1, containing 0.15 m*M* NaCl and 1 m*M* $MgCl_2$. These conditions were shown to be optimum for immobilization of thylakoid membranes. However, the preparation used here is mostly active at pH 6.0–6.5 (*24*). We thus used a Mes–NaOH buffer, pH 6.5, as a resuspending medium in order to analyze the effect of NaCl and $MgCl_2$ concentrations on the oxygen-evolution rates obtained before and after immobilization.

The optimum NaCl concentration for oxygen evolution was 1 m*M*, either for native photosystem II or when it was included in the immobilization medium (Table 1). Sodium chloride at 10 m*M* also gave good rates of oxygen evolution, but 100 m*M* was inhibitory. In the presence of $MgCl_2$, the highest rates were obtained at 0.5 m*M* before immobilization. However, the rates after immobilization were higher when 5 m*M* $MgCl_2$ was present in the immobilization medium (Table 1).

One of the important parameters in this study was the extent of activity remaining after immobilization. The recovery was greater in the presence of 5 m*M* $MgCl_2$ and 1 m*M* NaCl than in any other conditions used in Table 1. As seen in Table 1, nearly 40% of the initial activity was retained in the photosystem II immobilized under the conditions described above.

The presence of up to 40% of the initial electron-transfer activity in the immobilized material could justify its use in biotechnological applications if this material was more resistant to degradation than the native

Table 1
Oxygen Evolution Rates in Native and Immobilized Photosystem II as a Function of Salt(s) Concentration in the Resuspending Medium

Salt(s), m*M*		Oxygen evolution μmol O_2, (mg Chl·h)$^{-1}$	
		Native PSII	Immobilized PSII[a]
None		118	15 (13)
NaCl	0.1	158	13 (8)
	1	217	37 (17)
	10	201	35 (17)
	100	147	20 (14)
$MgCl_2$	0.5	185	35 (19)
	5	144	43 (30)
	50	101	14 (14)
NaCl	1		
+ $MgCl_2$	5	221	82 (37)

[a]The percentage of activity recovered after immobilization is given in parentheses.

preparation. We thus studied the effect of several treatments that are known to affect the functional integrity of this type of material.

In Fig. 1, the extent of functional deterioration under storage at 4°C is presented for native and immobilized photosystem II fractions in presence of either 1 m*M* NaCl, 5 m*M* $MgCl_2$, both 1 m*M* NaCl and 5 m*M* $MgCl_2$, or without salt. This experiment was also repeated with all the other salt concentrations listed in Table 1 (not shown). In general, although the initial activity was very much dependent on the ionic content of the medium (Table 1), the salt concentration of the immobilization medium did not significantly affect the storage stability in comparison to the differences obtained between immobilized and native samples. At least 80% of the activity found in the freshly immobilized material was maintained after 550 h of storage when NaCl, $MgCl_2$, or both NaCl and $MgCl_2$ were present in the immobilization medium. In contrast, the native material lost 75% of oxygen evolution after 100 h of storage.

After immobilization, the photosystem II fractions were also less sensitive to high pH than the native material (Fig. 2). The optimal pH for oxygen evolution was shifted from 6.0 to 6.5 upon immobilization. Furthermore, although almost all the activity was lost at pH 7.5 in untreated photosystem II, 50% of the maximal activity was still present in the immobilized samples (Fig. 2).

Another interesting reason for the use of immobilized materials is their resistance to elevated temperatures (*28*). The increased resistance of the immobilized photosystem II is shown in Fig. 3. After immobilization the maximal rate of oxygen evolution were found when the assay was

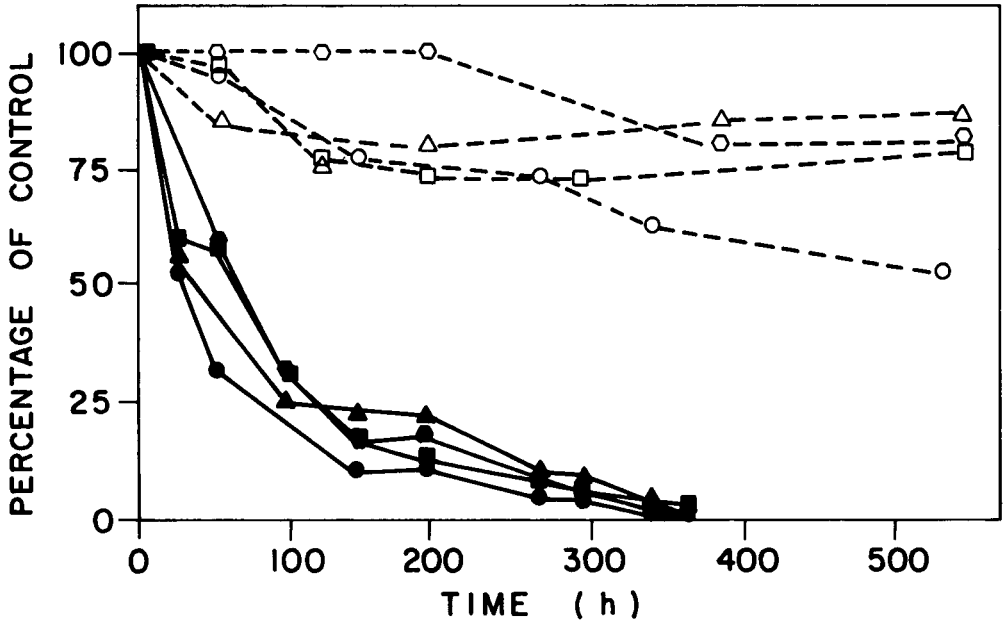

Fig. 1. Activity (i.e., O_2 evolution) of native and immobilized photosystem II as a function of storage time at 4°C with different concentrations of salts in the resuspending medium. The 100% values correspond to the rates given in Table 1. Closed symbols, native photosystem II; open symbols, immobilized photosystem II. ●, ○: no salt; ▲, △: 1 m*M* NaCl; ■, □: 5 m*M* $MgCl_2$; ⬢, ⬡: 1 m*M* NaCl and 5 m*M* $MgCl_2$; respectively.

performed at 35°C and the rates were similar at 20 and 50°C. On the other hand, a significant loss of activity was experienced with the native material when the temperature was elevated from 30 to 50°C (Fig. 3).

Since immobilized photosynthetic materials are likely to be used in photoreactors under high light intensities, their resistance to light exposure is of crucial interest. When thylakoids are exposed to strong light, the pigment photooxidation can be followed by monitoring the decrease in absorbance in the red region of the spectrum (*27*). The resistance of photosynthetic pigments to light exposure was clearly improved by the immobilization procedure. In the conditions used in Fig. 4, 75% of the pigments absorbing at 680 nm were bleached after about a 20-min illumination. In comparison, the pigments in immobilized photosystem II were only slightly affected by photooxidation. Electron transfer was also protected against photodamaging after immobilization. This was shown by measuring oxygen evolution after a given time of strong light treatment that causes the so-called photoinhibition of electron transfer (*29*). With the light intensity used, only 8 min of light exposure was needed for complete photoinhibition of the native material (Fig. 5). In contrast, not more than 50% of inhibition was found in immobilized samples, even after a 15-min light treatment (Fig. 5).

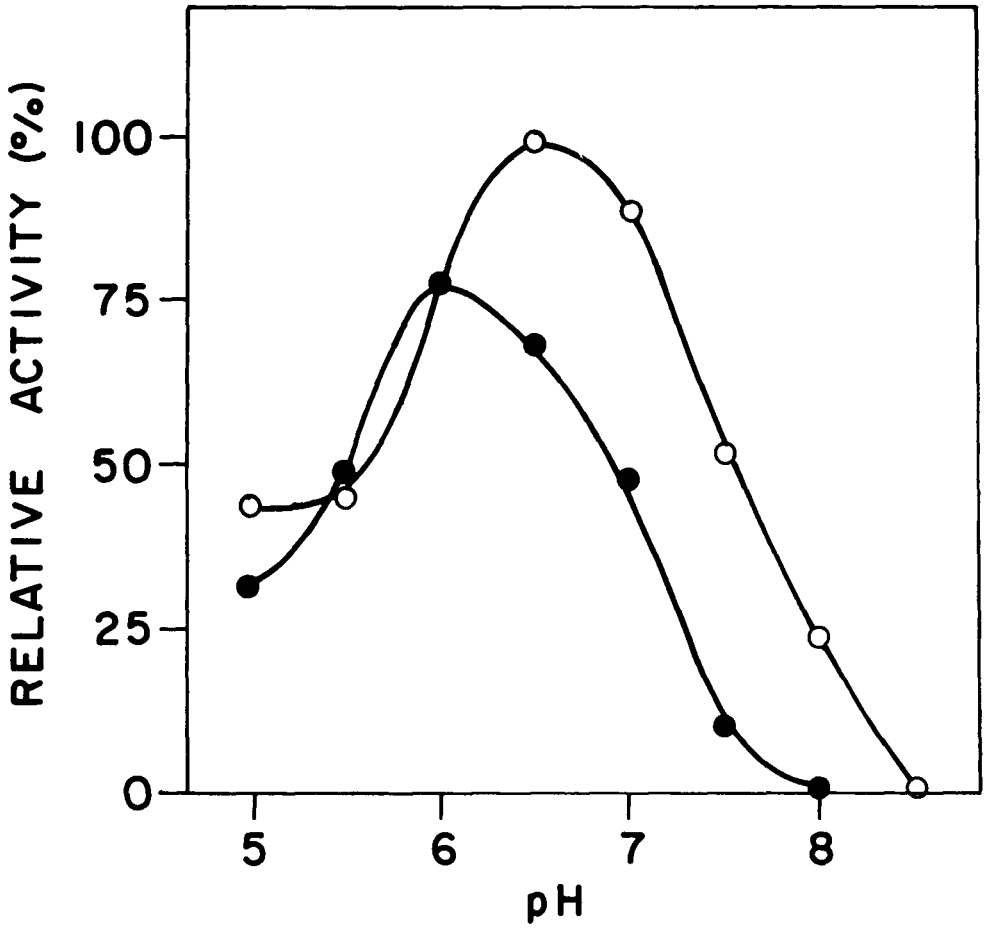

Fig. 2. Effect of pH in the assay medium on oxygen-evolution rates. The traces were normalized at pH 6.0. ●, native photosystem II with a maximal rate at pH 6.0 of 242 μmol O_2 (mg Chl h)$^{-1}$; ○, immobilized photosystem II with a maximal rate at pH 6.5 of 93 μmol O_2 (mg Chl h)$^{-1}$.

DISCUSSION

Part of the initial activity in native thylakoid membranes is lost upon their immobilization in glutaraldehyde–albumin cross-linked matrix (*18,23*). Nevertheless, this treatment is still of great interest because the remaining activity is stabilized to a significant extent. In this work, we demonstrated that a Triton X-100-derived photosystem II submembrane fraction can be immobilized following the above procedure. The activity remaining after the immobilization treatment (40%) was as high as in whole thylakoids when the optimum conditions were used (5 m*M* $MgCl_2$, 1 m*M* NaCl) (Table 1).

It is noteworthy that the loss of apparent activity after immobilization does not necessarily imply a decreased integrity of the entrapped

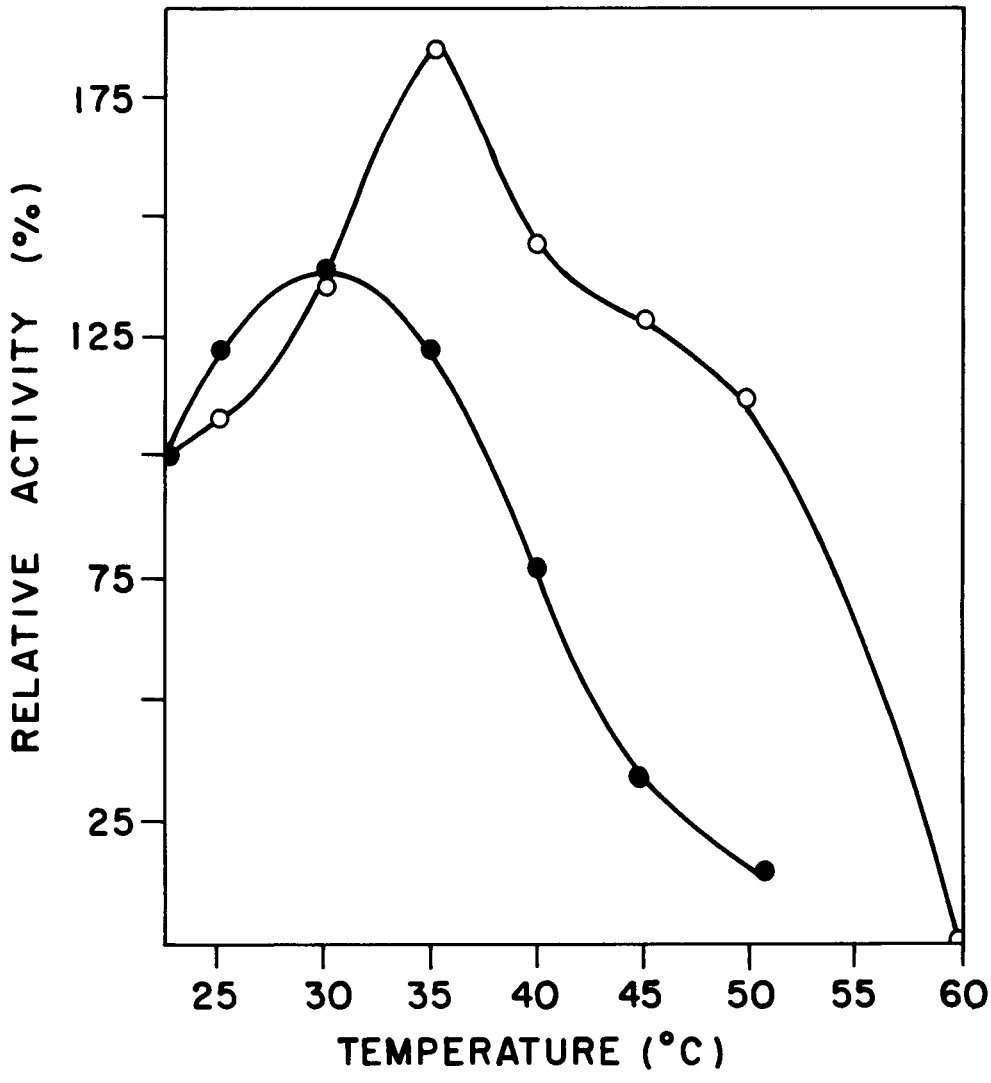

Fig. 3. Effect of temperature in the assay medium on oxygen-evolution rates. The traces were normalized at 22.5°C when the activities were 262 μmol O_2 (mg Chl h)$^{-1}$ for native photosystem II (●) and 100 μmol O_2 (mg Chl h)$^{-1}$ for immobilized photosystem II (○).

membranes. Even if the exact mechanism of protein cross-linking is not fully understood, it was shown by electron micrography that immobilized thylakoids preserve their structural integrity after the glutaraldehyde matrix has formed (*23*). Glutaraldehyde by itself is known to inhibit electron transfer between photosystem II and photosystem I (*17,30*). However, photosystem I is virtually absent in our preparation. Addition of glutaraldehyde to photosystem II fractions in solution with 2,6-dichlorobenzoquinone as electron acceptor did not affect oxygen evolution (the authors observations). Thus, we postulate that the loss of activity

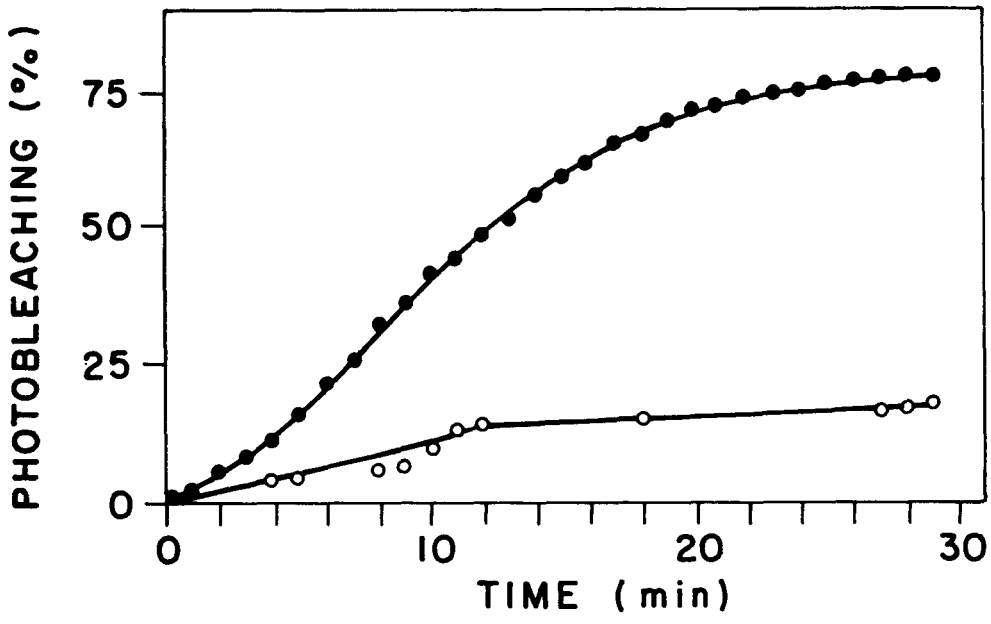

Fig. 4. Photobleaching kinetics at 680 nm. The initial rates (percent/min) were 2.5 for native photosystem II (•) and 0.7 for immobilized photosystem II (○). After 3.5 min of illumination the photobleaching rate of native photosystem II repetitively increased by a factor of two.

after immobilization is rather the result of a diffusion barrier imposed by the matrix (*31*). This barrier could limit the access of exogenous electron acceptors and of water to their specific sites and/or the release of oxygen from the water-splitting complex. Furthermore, the membrane microviscosity is likely to increase following entrapment within the glutaraldehyde matrix. This assertion is supported by the possibility of covalent bonding between glutaraldehyde molecules and the photosynthetic membrane (*31*). This phenomenon could decrease platoquinone access to its site at the Q_B protein and, possibly, the oxygen-evolving process by itself.

The highest yield of activity recovered after glutaraldehyde treatment was found when $MgCl_2$ was present at a concentration of 5 m*M* (Table 1). However, the optimal $MgCl_2$ concentration for photosystem II in solution was 0.5 m*M*. The negative effects of the immobilization procedure were thus reduced by an increased level of Mg^{2+}. The results presented here do not provide an explanation for this effect. However, a possible interpretation would reside in the screening of membrane surface charges by Mg^{2+}, which is known to increase membrane stacking. In presence of 5 m*M* $MgCl_2$, the reduced access of glutaraldehyde molecules into the oppressed regions will consequently diminish the effect of immobilization on membrane viscosity.

The experiments presented also demonstrated that immobilized photosystem II was less sensitive to long-term storage at 4°C, high pH

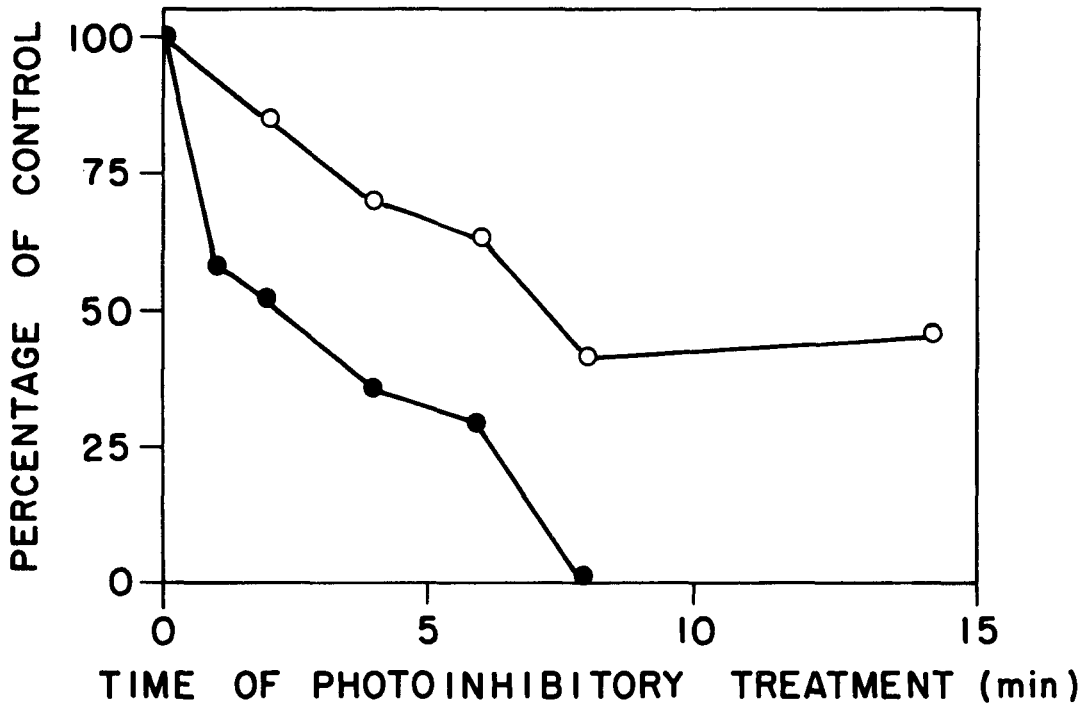

Fig. 5. Photoinhibition of electron transfer in photosystem II. The initial rates (percent/min) were 35 for native photosystem II (●) and 7.5 for immobilized photosystem II (○).

and temperature, and strong illumination (Figs. 1–5). The increased membrane microviscosity and the decreased access of oxygen to the photosynthetic components are probably responsible for the increased stability. Part of the resistance to pigment photobleaching and to photoinhibition of electron transfer could also indicate that at an equal light intensity, the immobilized membranes absorb less light energy than the native ones because of the strong light dispersion induced by the matrix (*31*). This later phenomenon could also lead to lower oxygen-evolution rates in the immobilized material.

CONCLUSION

In conclusion, we have shown that the immobilization procedure can be successfully applied to submembrane fractions to increase their functional stability. This type of preparation will be studied in our laboratory in a photoelectrochemical cell.

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