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Immobilized Thylakoids in a Cross-Linked Albumin Matrix

EFFECTS OF CATIONS STUDIED BY ELECTRON MICROSCOPY, FLUORESCENCE EMISSION, PHOTOACOUSTIC SPECTROSCOPY, AND KINETIC MEASUREMENTS

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ABSTRACT

Immobilization of lettuce (*Lactuca sativa*) thylakoids has been performed by using glutaraldehyde and bovine serum albumin. Confirming previous reports, a stabilization of the O_2 evolution activity of the photosystem II (PSII) under storage and functional conditions has been observed. The present work is devoted to the role played by mono-and divalent cations, during the immobilization process itself, on the O_2 production. Four types of measurements have been employed: kinetic measurements, low temperature (77 K) fluorescence emission, photoacoustic (PA) spectroscopy, and electron microscopy observations. We show that the effect of glutaraldehyde is complex because it acts as an inhibitor, a stabilizing agent, and a cross-linking reactive. In the present studies, the thylakoids are immobilized within a polymeric insoluble albumin matrix. The highest activity yield and the best storage conditions are obtained when 0.15 mM Na^+ (or K^+), 1 mM Mg^{2+}, and 0.1 mM Mn^{2+} are present in the resuspending media before the immobilization. Due to modifications of the ionic content during such a process, structural differences are observed on the stacking degree of thylakoids. No modification of the fluorescence and PA spectra after the immobilization are found. Furthermore, a correlation between activities and spectral changes have been shown: when the activities increase, the F_{735} to F_{695} ratio increases and the PA_{676} to PA_{440} ratio decreases.

In recent years, there has been progress (3, 16, 23, 32) in the research on technological applications of photobiological solar energy conversion (biophotolysis of water, photohydrogen production, and ATP regeneration). However, the life time of the isolated chloroplasts was very short, and the stability of PSI and PSII activities over a long period of time was a crucial limitation for these applications.

Recent advances have been made in techniques for immobilization of biocatalysts (27), and chloroplasts isolated from plants were immobilized by several different methods like microencapsulation (22), adsorption (33), entrapment within gels (9, 14, 21, 29), radiation polymerization (13), or cross-linking with glutaraldehyde in the presence of albumin (9, 20, 23). A functional stabilization of the photosystems activities has been obtained and some of these methods were recently compared (9). In the present paper, we have selected the cross-linking technique to study the effect of mono- and divalent cations on the PSII stability.

The action of glutaraldehyde on the structure and function of chloroplast has been studied extensively (17, 18, 38) and was reviewed recently by Papageorgiou (30). The glutaraldehyde fixation stabilizes activities but blocks chloroplast reactions at specific sites (17). However, some microconformational changes may still occur after fixation (18, 30). In such studies, the glutaraldehyde treatment does not give insoluble and polymeric structures. In our work, the co-crosslinking method gives thylakoids immobilized within an insoluble albumin matrix. Our aim is to obtain information about the role played by mono- and divalent cations, during the immobilization process itself, on the PSII-dependent O_2 evolution of immobilized thylakoids.

The effect of ions in light-driven processes on the photosynthetic electron transport and on the ultrastructure of the chloroplast thylakoid membranes has been widely studied (2, 4, 19). By using fluorescence techniques, several authors (2, 8, 10, 15, 26, 37) have pointed out that mono- and divalent cations concurrently affect the excitation energy distribution from PSII to PSI. It has been also demonstrated that the stacking of thylakoid membranes was reversibly modified *in vitro* by changing the salt composition (19).

Since the immobilization process leads to a modification of the environment around thylakoid membranes, the studies have to be performed by using not only kinetic measurements but also spectral and structural investigations. Especially, we use the PA[1] spectroscopy (1, 5, 6, 25, 31) to obtain information about the nonradiative deexcitation processes that occur in a system, after it has been optically excited. It is known (5, 25) that changes in PA signal reflect the light-induced changes into heat and consequently indicate the efficiency of the photochemical reactions. On the other hand, immobilized material consisting of solid, insoluble, and amorphous particles are optically unfit for classical transmission or relection spectrometry (35, 36).

In this report, we present kinetic, structural (electron microscopy observations), and spectral (low-temperature fluorescence emission and PA spectroscopy) investigations. The purpose of our work is to determine the optimal conditions in the use of mono- and divalent cations in the resuspending media before the immobilization process. Our aim is to obtain the best results in the storage stability (at 4°C in absence of light) and in the functional stability (continuous use at 20°C under illumination) of immobilized thylakoids.

MATERIALS AND METHODS

Thylakoid Preparation. Intact chloroplasts were isolated from lettuce leaves (*Lactuca sativa*), obtained from a local market,

[1] Abbreviation: PA, photoacoustic.

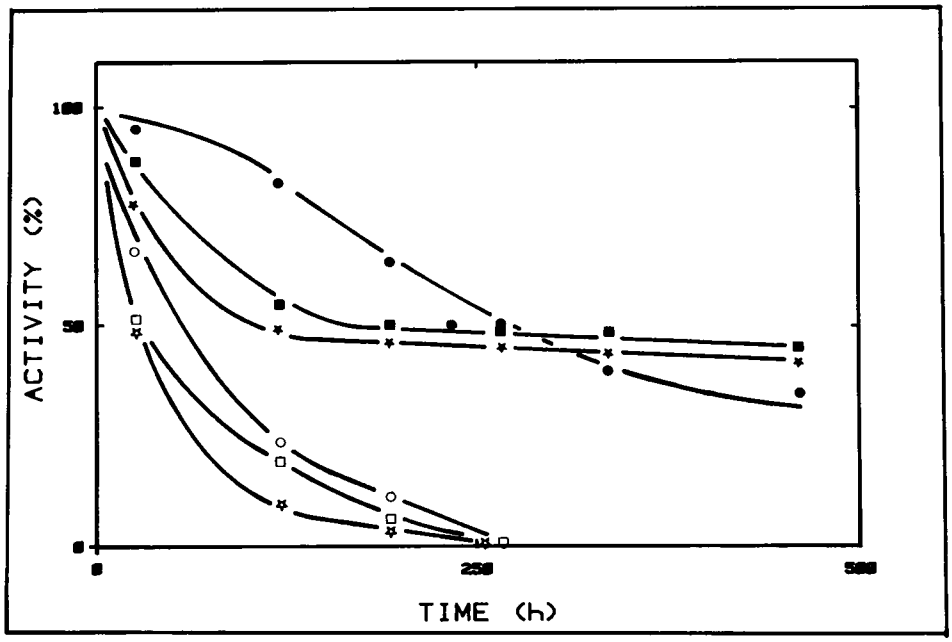

FIG. 1. Activity of native and immobilized thylakoids as a function of time in storage in the dark at 4°C. Several Na^+ concentrations were used. The 100% values correspond to: 174 μmol O_2 mg^{-1} Chl h^{-1} for native thylakoids with 0 mM Na^+ (○); 162 μmol O_2 mg^{-1} Chl h^{-1} for native thylakoids with 0.15 mM Na^+ (□); 158 μmol O_2 mg^{-1} Chl h^{-1} for native thylakoids with 1.5 mM Na^+ (☆); 33 μmol O_2 mg^{-1} Chl h^{-1} for immobilized thylakoids with 0 mM Na^+ (●); 71 μmol O_2 mg^{-1} Chl h^{-1} for immobilized thylakoids with 0.15 mM Na^+ (■); 57 μmol O_2 mg^{-1} Chl h^{-1} for immobilized thylakoids with 1.5 mM Na^+ (★).

Table I. *Effect of Na^+ and K^+ on PSII Activity, Fluorescence Ratio, and Photoacoustic Ratio of Native and Immobilized Thylakoids*

	Ion Concn.	Activity	$\frac{F_{735}}{F_{695}}$	$\frac{PA_{676}}{PA_{440}}$
	mM	*μmol O_2 mg^{-1} Chl h^{-1}*	*ratio*	
Na^+				
Native thylakoids	0	174	2.7	0.82
	1.5	158	2.5	0.85
Immobilized thylakoids	0	33	1.9	0.84
	1.5	57	2.0	0.76
K^+				
Native thylakoids	0	157	2.5	0.80
	1.5	150	1.8	0.79
Immobilized thylakoids	0	43	1.8	0.84
	1.5	54	2.0	0.75

according to Epel and Neumann (12). The blending medium contained 0.33 M sorbitol, 10 mM $Na_4P_2O_7$, 20 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, and 0.15 mM KH_2PO_4/K_2HPO_4, adjusted to pH 6.8. After centrifugation (3000g, 30 s), the thylakoids are obtained by osmotic shock, centrifuged again, and resuspended in 0.33 M sorbitol, 0.15 mM KH_2PO_4/K_2HPO_4, 50 mM Hepes, 1 mM $MgCl_2$, 0.5 mM EDTA, 1% BSA, adjusted to pH 7.6.

Immobilization Method. As previously described (9), a solution was produced by mixing 1.65 ml of 0.05 M phosphate buffer (pH 7.1), 1.25 ml of 20% BSA solution, 0.6 ml of thylakoid suspension (corresponding to 2 mg of Chl) and 1 ml of glutaraldehyde solution at 1.5%. This mixture was frozen at −20°C during 2 h and then slowly thawed to 4°C. Due to the freezing-thawing process, an insoluble proteic phase was obtained. The green, sponge-like structure which exhibited good mechanical properties was then rinsed in a distilled water flow.

Oxygen Production. O_2 evolution was measured polarographically with a Clark-type electrode. The temperature was maintained at 20°C. Reaction media contained the suspending buffer, 5 mM $MgCl_2$, 5 mM NH_4Cl (as the uncoupling agent), and were illuminated at a saturating light. *p*-Benzoquinone (1 mM) was used as the electron acceptor. The activity is expressed in μmol O_2 mg^{-1} Chl h^{-1}. The activity yields (*i.e.* fractional retention of O_2 production activity on immobilization) are obtained by comparing values in suspension and after immobilization, for the same content of Chl. For storage stability studies, native and immobilized thylakoids were stored in the dark at 4°C, and samples were periodically assayed as described above. For functional stability studies, native and immobilized thylakoids were continuously monitored at 20°C under illumination.

Fluorescence Emission Measurements. The fluorescence emission spectra were recorded at 77 K using a laboratory-built apparatus described by Sironval *et al.* (34). The excitation wavelength was 436 nm (light from a Hg vapor lamp isolated through an interference filter). Native and immobilized thylakoids were deposited on a copper support and immediately cooled with liquid N_2 in a special holder (34).

Photoacoustic Experiments. The laboratory-built two beam PA spectrometer was previously described (36). Light modulation frequency was 36 Hz. PA signals were sent to a HP 2649G graphic terminal through a 12 bit analog digital converter. According to Cahen *et al.* (5), the spectra can be normalized at 440 nm to facilitate the comparison of the different samples.

Electron Microscopy. Native and immobilized thylakoids were fixed with 3% glutaraldehyde and 2% OsO_4 and were then dehydrated and embedded in Epon. Ultrathin sections were made with a LKB ultrotome III and were contrasted with uranyl acetate and lead citrate. The micrographs were obtained using a JEOL 100C electron microscope.

RESULTS

In the present work, the effect of cations on immobilized thylakoids is always studied by varying the addition of cations

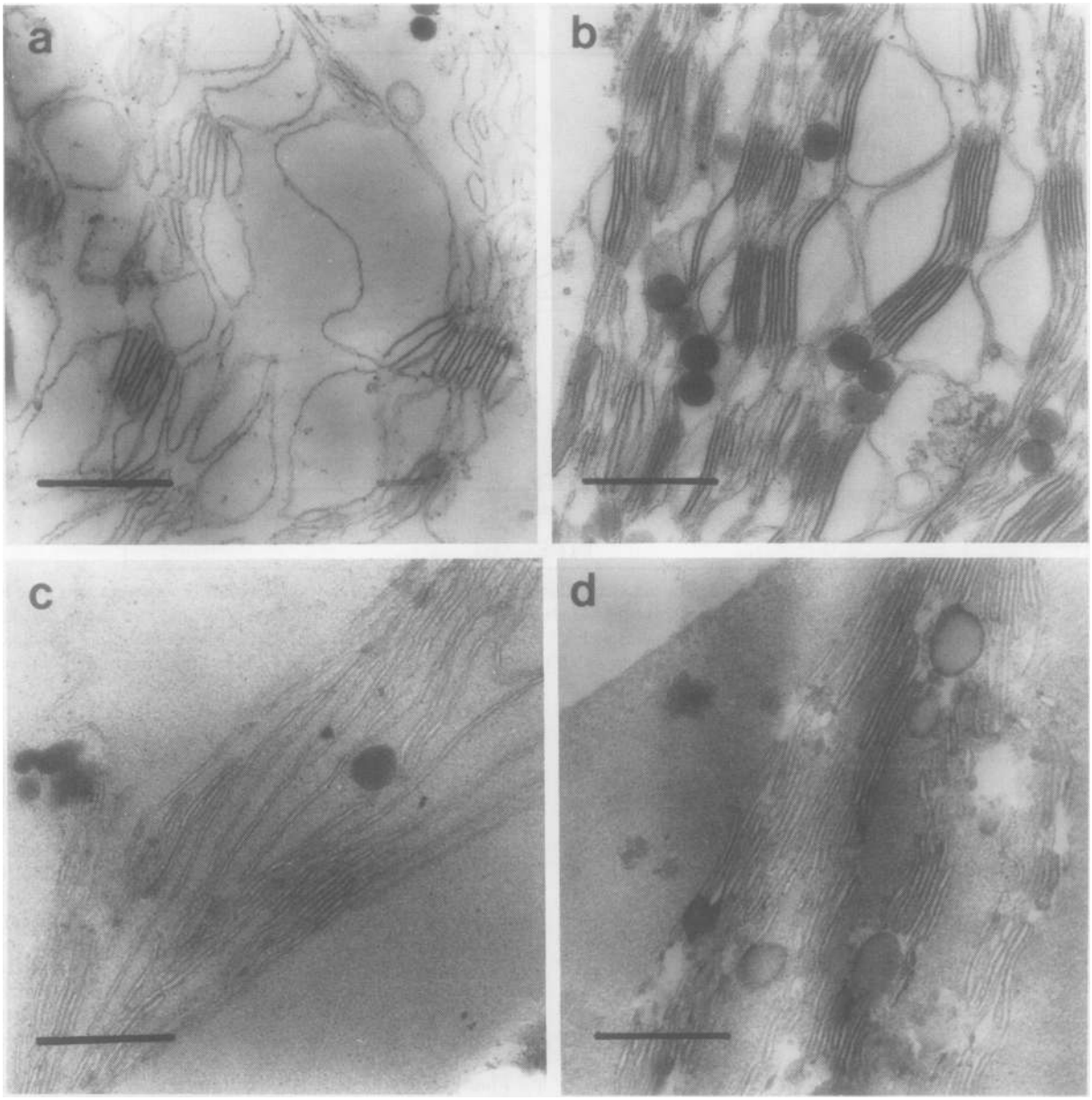

FIG. 2. Ultrathin sections of native thylakoids prepared in the absence (a) or in the presence of 1.5 mM of Na^+ (b) and immobilized thylakoids in albumin matrix prepared without (c) or with 1.5 mM of Na^+ (d). Bar, 0.5 μm.

before the immobilization.

Influence of Monovalent Cations. Thylakoids were resuspended in the Hepes-sorbitol buffer pH 7.6 containing 0 to 1.5 mM Na^+ (or K^+) from Na_2HPO_4/NaH_2PO_4 (or K_2HPO_4/KH_2PO_4). The other salts were the same as in the standard medium (which contains divalent cations). No significant variation of the O_2 production has been observed when Na^+ (or K^+) are present from 1.5 μM to 0.15 mM in native as well as in immobilized thylakoids. The most satisfactory results for the activity yield after immobilization (see "Materials and Methods") have been obtained with 0.15 mM Na^+ (43.5%) or 0.15 mM K^+ (50%). It is well known that a decrease in activity is due to the chemical effect of the glutaraldehyde action (38). However, as already described (17, 38) in fixation studies, we observe also a stabilization of cross-linked thylakoids. Figure 1 illustrates the time course of changes of the PSII activity of native and immobilized thylakoids, when they are prepared with or without 0.15 to 1.5 mM Na^+, during storage at 4°C. The immobilized thylakoids (closed symbols) retain PSII activity longer than native thylakoids (open symbols). The corresponding activities are given in the legend of Figure 1. Even after 480 h storage, 50% of the initial activity remains when 0.15 mM Na^+ is present before the immobilization. In the same manner, it was verified that the functional stability (O_2 production continuously monitored under illumination) is also improved.

The ultrastructure of native and immobilized thylakoids in albumin matrix prepared without or with 1.5 mM Na^+ (in Hepes-sorbitol buffer pH 7.6) is shown in Figure 2. Without Na^+ the thylakoid membranes in suspension appear largely unstacked (Fig. 2a) and a high degree of stacking is evident with 1.5 mM Na^+ (Fig. 2b). However, no dramatic change in membrane appression occurs in the corresponding immobilized thylakoids (Fig. 2c and d). It seems that a high degree of stacking is restored by the immobilization process itself (9).

Fluorescence emission and PA spectra of both native and immobilized thylakoids have been recorded. In the two cases, the positions of maxima were not modified by the immobilization

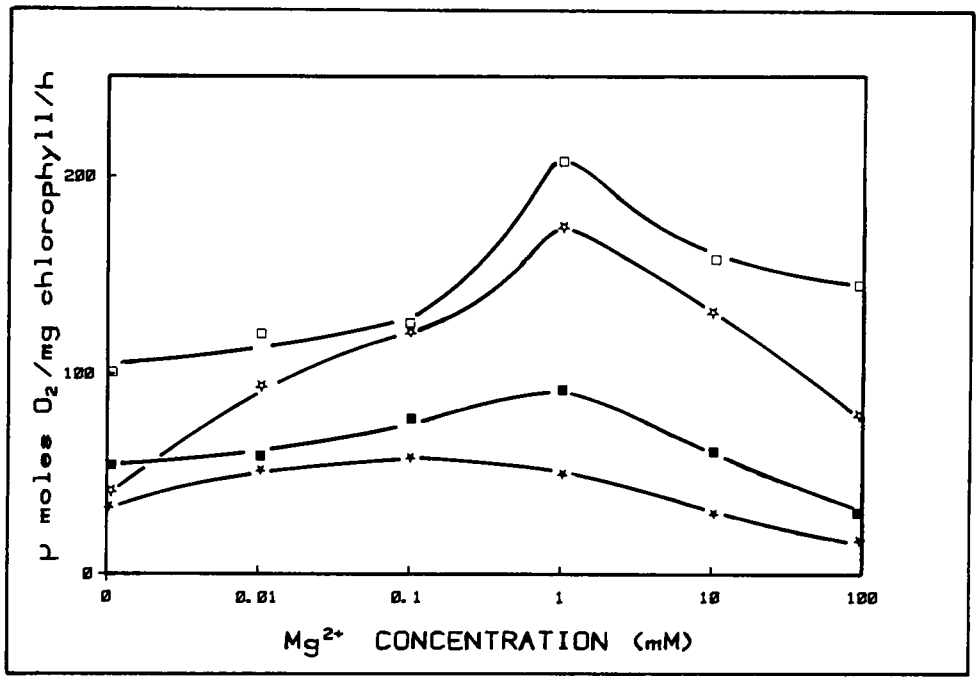

FIG. 3. O_2 evolution measured as a function of the Mg^{2+} concentration used during the thylakoids preparation. Benzoquinone was used as electron acceptor for native (□) and immobilized thylakoids (■). Methyl viologen was used as electron acceptor for native (☆) and immobilized thylakoids (★).

process (see below for the divalent cation effect on the fluorescence and PA spectra). The ratios of fluorescence intensity emitted at 735 nm (F_{735}) to that at 695 nm (F_{695}) have been used for evaluating the energy transfer from PSII to PSI (Table I). The highest values have been obtained for native thylakoids. We have observed a slight decrease of the energy transfer capacity for immobilized thylakoids. That decrease is due to the immobilization process, since in the present studies, changes in reabsorption of fluorescence are insignificant given the low Chl content of native and immobilized thylakoids. As already described by Cahen *et al.* (5), it is possible to calculate the ratio of PA signal at 676 nm (PA_{676}) to that at 440 nm (PA_{440}) (Table I). The increase of such a ratio indicates increased heat generation (5, 6, 31), *i.e.* a decrease of the photochemical activity. By the nature of the PA effect (5, 6, 25), changes in the signal are not to be related to light scattering. The PA signal ratio obtained for immobilized thylakoids seems to be slightly lower (0.75 *versus* 0.78) than that of native thylakoids. A correlation between activities and spectral changes measured by fluorescence emission and PA spectroscopy can be noted (Table I). For native thylakoids, the activities are lower for high ionic concentrations the F_{735} to F_{695} ratio decreases and the PA_{676} to PA_{440} ratio increases as a function of Na^+ and remain unchanged for K^+. For immobilized thylakoids, such parameters change in the opposite direction: activities and fluorescence ratio increase and PA ratio decreases as a function of Na^+ or K^+ concentration used before immobilization.

Influence of Divalent Cations. The effects of Mg^{2+}, Mn^{2+}, and Ca^{2+} (present in the resuspending medium) on PSII activity were investigated on native and immobilized thylakoids. $MgCl_2$ concentrations were varied from 0 to 100 mM (in the presence of 0.15 mM K^+, 1 mM $MnCl_2$, and 2 mM EDTA in Hepes-sorbitol buffer pH 7.6). Figure 3 shows the changes in the O_2 production of native (open symbols) and immobilized (closed symbols) thylakoids as a function of Mg^{2+} concentration. A maximum in the O_2 production is observed for 1 mM Mg^{2+}. However, after immobilization the phenomenon is not so important. When the activity of both the photosystems (PSI and PSII) is recorded (by using methyl viologen), the same features occur (Fig. 3).

When 1 mM Mg^{2+} is present in the resuspending media, the activity yield after immobilization is about 45% (92 μmol O_2 mg^{-1} Chl h^{-1} compared to 208 μmol O_2 mg^{-1} Chl h^{-1}: see Fig. 4a). The decay of PSII activity of native and immobilized thylakoids prepared in the absence or with 1 mM or 100 mM $MgCl_2$, in storage conditions at 4°C, is illustrated on Figure 4a. The use of 1 mM $MgCl_2$, in both cases is optimal for the remaining of the activity. The PSII activity of native thylakoids disappears completely after 300 h of storage, while the PSII activity of immobilized thylakoids retained 35% of the original value after 480 h (and 20% even after 800 h). The same features have been also observed after a continuous use under illumination at 20°C.

When thylakoids were resuspended in the presence of low Mn^{2+} concentration (0.01.–0.1 mM) and in the presence of Mg^{2+} and EDTA, no significant variation of the 0_2 production occurs (see the legend of Fig. 4b). The same behavior is observed for native and immobilized thylakoids. Figure 4b shows the curves of PSII activity as a function of storage time for the thylakoids prepared with 0.01 or 1 mM $MnCl_2$. The activity of immobilized thylakoids remains far longer than that of native ones, and 30% of the original value is retained after 580 h. The presence of a low concentration of Mn^{2+} is, however, necessary to obtain substantial activity and stability (8). When Ca^{2+} is used instead of Mn^{2+}, the same features are observed (results not shown here).

The removal of Mn^{2+} (or Mg^{2+}) in the resuspending media of native thylakoids leads to unstacked and unfolded membranes (Fig. 5a). The extensively restacked membranes are observed with an excess of ions (Fig. 5c). An intermediate situation is shown in the presence of 10 mM Mn^{2+} (or Mg^{2+}) in Figure 5b. As shown for monovalent cations (Fig. 2, c and d), the ultrastructure changes in the immobilized thylakoids are not so pronounced (Fig. 5, d and e). However, the restacking is more significant when an excess of Mn^{2+} (or Mg^{2+}) is present (Fig. 5e).

The 77 K fluorescence spectra of native and immobilized thylakoids (prepared without or with 100 mM $MgCl_2$) exhibit normal peaks at 685, 695, and 735 nm (Fig. 6, a and b). The fluorescence ratios (F_{735}:F_{695}) are given in Table II and can be compared to the corresponding PSII activities. For native and immobilized thyla-

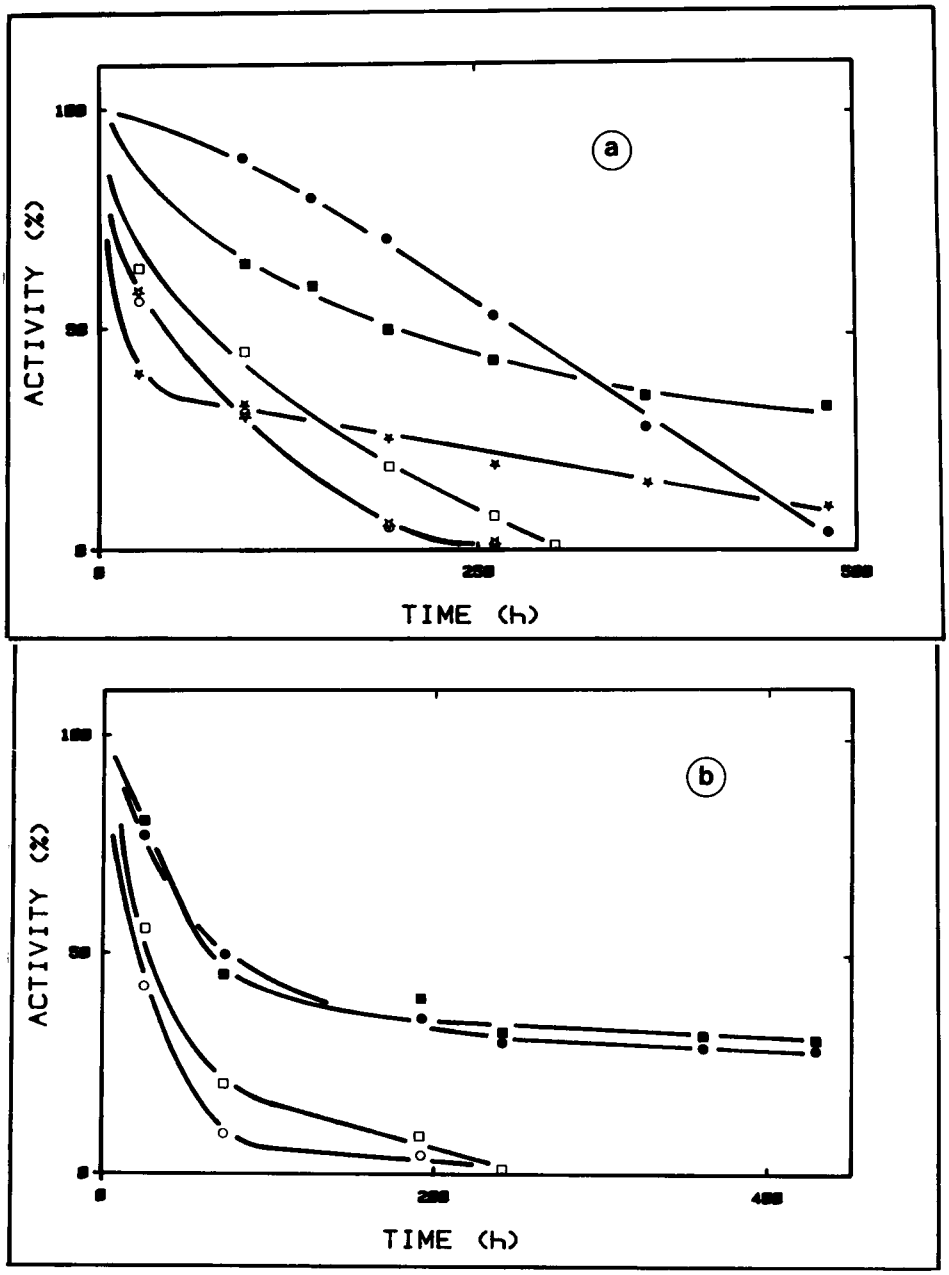

FIG. 4. a, Activity of native and immobilized thylakoids as a function of time in storage in the dark at 4°C. Several Mg^{2+} concentrations were used. The 100% values correspond to: 102 μmol O_2 mg^{-1} Chl h^{-1} for native thylakoids in absence of Mg^{2+} (○); 208 μmol O_2 mg^{-1} Chl h^{-1} for native thylakoids with 1 mM of Mg^{2+} (□); 146 μmol O_2 mg^{-1} Chl h^{-1} for native thylakoids with 100 mM of Mg^{2+} (☆); 56 μmol O_2 mg^{-1} Chl h^{-1} for immobilized thylakoids in absence of Mg^{2+} (●); 92 μmol O_2 mg^{-1} Chl h^{-1} for immobilized thylakoids with 1 mM of Mg^{2+} (■); 30 μmol O_2 mg^{-1} Chl h^{-1} for immobilized thylakoids with 100 mM of Mg^{2+} (★). b, Activity of native and immobilized thylakoids as a function of time in storage in the dark at 4°C. Several Mn^{2+} concentrations were used. The 100% values correspond to: 240 μmol O_2 mg^{-1} Chl h^{-1} for native thylakoids with 0.01 mM of Mn^{2+} (○); 230 μmol O_2 mg^{-1} Chl h^{-1} for native thylakoids with 1 mM of Mn^{2+} (□); 100 μmol O_2 mg^{-1} Chl h^{-1} for immobilized thylakoids with 0.01 mM of Mn^{2+} (●); 75 μmol O_2 mg^{-1} Chl h^{-1} for immobilized thylakoids with 1 mM of Mn^{2+} (■).

koids, a decrease of these ratios occurs as a function of the Mg^{2+} (or Mn^{2+}) concentration (between 1 and 100 mM), concomitantly with the decay of the PSII activity (Table II). However, the respective ratio between PA signals at 676 and 440 nm increase as a function of divalent cation concentration confirming the inhibitory effect of the excess ion. As could be seen in the respective spectra (Fig. 7, a and b), the amplitude of the phenomenon is greater for native thylakoids. However, when Mn^{2+} excess is used before immobilization, the activity is equal to zero; due to spectral modifications, the PA_{676} to PA_{440} ratio cannot be measured (Table II).

DISCUSSION

The immobilization of thylakoid membranes in an insoluble albumin-glutaraldehyde matrix is very convenient for applications in relation to the chemical conversion of solar energy. As already observed in the case of suspensions (17, 18, 38), the glutaraldehyde acts not only as a chemical inhibitor of the electron transport activity but also as a stabilizing agent. However, in the present report, the stability observed under storage and functional conditions is increased by the concomitant presence of serum albumin. Especially, BSA has been shown as an important factor determin-

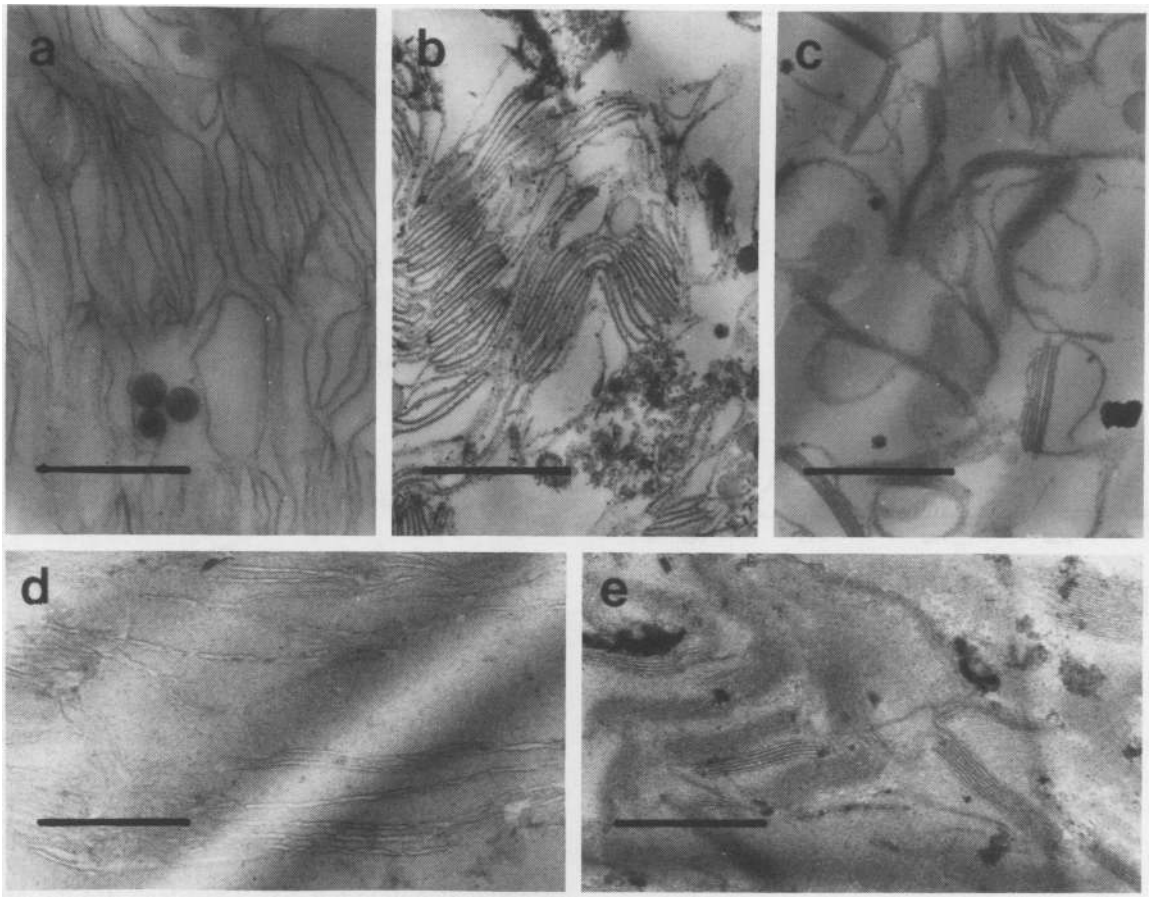

FIG. 5. Ultrathin sections of native thylakoids prepared in the absence (a) or in the presence of 10 mM (b) and 100 mM (c) of Mn^{2+}, and immobilized thylakoids prepared without (d) or with 100 mM of Mn^{2+} (e) Bar, 0.5 μm.

ing the thylakoid stability (28). To elucidate further the parameters which can be critical for such a stability, we have characterized in the present work the effect of mono- and divalent cations during the thylakoid preparations before the immobilization itself.

Despite the stabilization of the structure observed by electron microscopy (Fig. 2, c and d; to Fig. 5, d and e), we have clearly shown that a loss of PSII activity occurs just after immobilization (Tables I and II). However, the highest activity yield and the best storage conditions have been obtained when 0.15 mM Na^+ and 1 mM Mg^{2+} are present respectively in the resuspending media (Figs. 1 and 4a), the optimal Mn^{2+} concentration being 0.1 mM. Such results indicate that the medium composition of the thylakoid suspension should be clearly stated to optimize the immobilization conditions.

The ultrastructure of thylakoids observed without or treated with mono- and divalent cations (Figs. 2, a and b; Fig. 5, a–c) agrees with previous results of Gross and Prasher (15). The apparent contradictions with the observations of Izawa and Good (19) can be explained by the existence, in our conditions, of a substantial ionic strength without Na^+ or Mg^{2+} (because 1 mM Mg^{2+} or 0.15 mM Na^+ are present, respectively). The grana appear slightly more stacked in the absence of Na^+ (Fig. 2a) than in the absence of Mg^{2+} (Fig. 5a) because in this last case the low concentration of Na^+ (present) causes unstacking. Due to modifications of the ionic content during the immobilization process, structural differences can be observed on the stacking degree of the thylakoids as compared to the native ones. Especially, our investigations show that the restacking effect observed for the immobilized thylakoids is more important in the absence of Na^+ or Mg^{2+} than in its presence (*cf.* Figs. 2, a and c with Figs. 5, a and d). Such results can be clearly explained by the liquid-solid phase separation which occurs during the insoluble matrix production. The phenomenon is obviously accompanied by an increase of the local ion concentration within the cross-linked albumin phase. Nevertheless, the behavior of alginate-immobilized thylakoids (9, 14) as a function of ion concentration should be quite different. In such a case, the matrix structure is composed of numerous, large cavities containing the entrapped biological material (24) and no steric constraints can be observed.

Isaakidou and Papageorgiou (18) have shown, in the case of the PSII, that after glutaraldehyde treatment (in suspension), unstacked thylakoids exhibit higher activities than stacked ones. In such a case, the inhibition is complete for the PSI. In our case, the activities after immobilization are not substantially different when the thylakoids are prepared with or without cations (Tables I and II). However, the stabilities under storage conditions are quite different (Figs. 1 and 4a). Duniec *et al.* (11) have shown that changes of pH in chloroplast suspension induces an exchange between metal ions and H^+ and consequently a spacing modification between membranes in grana stacks occurs. Due to the constraints inside the matrix and the increase of local ion concentration, such changes cannot occur after immobilization. The extensive stacking observed after immobilization is not responsible for the increase of the activities but might be related to the stabilization effect. It is noteworthy that diffusional limitations are very important and the diffusion of the electron acceptor inside the matrix can partly govern the kinetics.

A correlation between activities and spectral changes has been shown. The native thylakoids in the absence of Na^+ or K^+ (in the presence of 1 mM Mg^{2+}) exhibit a F_{735} to F_{695} ratio lower (2.7 or 2.5) than in the absence of Mg^{2+} (but in the presence of 0.15 mM Na^+) (4.4) and higher than in the presence of Mg^{2+} excess (2)

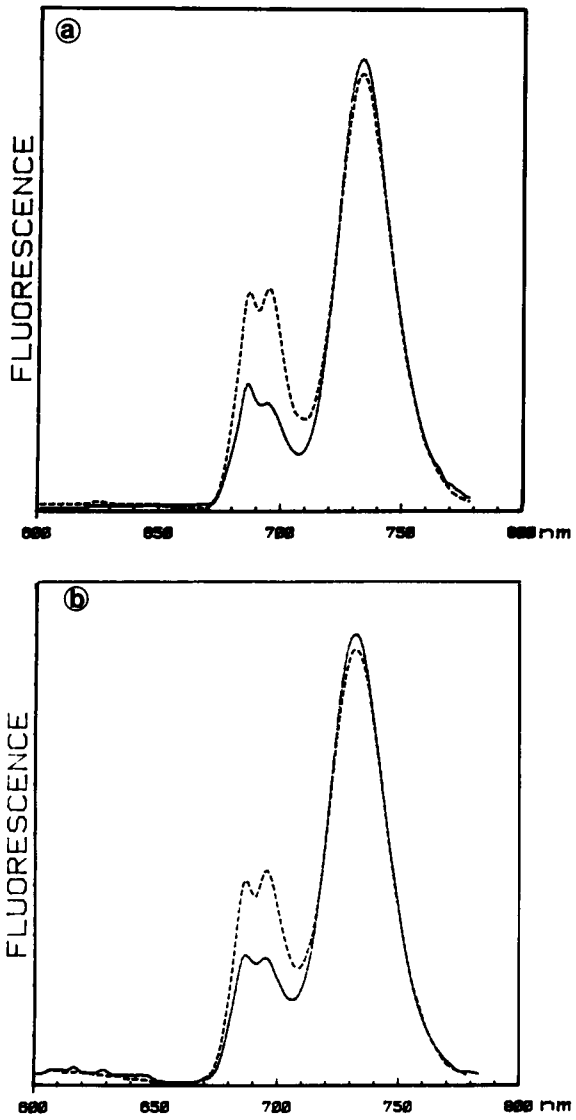

FIG. 6. Low temperature (77 K) fluorescence emission spectra of native (a) and immobilized (b) thylakoids prepared in the absence (——) or in the presence (– – –) of 100 mM of Mg^{2+}.

(Tables I and II). When the F_{735} to F_{695} ratio is high (*i.e.* when the absorbed excitation is preferentially distributed to PSI), we have shown that the PA_{676}/PA_{440} ratio is generally low and the activities high. In such a case, the conversion of light into heat corresponds to a minimal level and there is a high efficiency of the photosynthetic activities. Especially, for native thylakoids the influence of K^+ on the PA_{676} to PA_{440} ratio is not significant, but the addition of an excess of Mg^{2+} leads to an increase of such a ratio (and a decrease of the fluorescence ratios). The fluorescence results agree with the observations of Wong *et al.* (37) which indicate that at a neutral pH the divalent cations cause a decrease of the F_{735} to F_{695} ratio (in our case, due to low pH variations, changes in fluorescence as a function of pH are insignificant). However, some modifications of this ratio occur as a function of the ionic content used before immobilization. Especially, a decrease is observed upon the addition of Mg^{2+} (or Mn^{2+}) both with native and immobilized thylakoids. Mohanty *et al.* (26) have shown that this effect is absent in isolated thylakoids fixed with glutaraldehyde. But, in their experiments, contrary to ours, Mg^{2+} was always added after the fixation, not before. Likewise, immobilized thylakoids incubated with ions, after immobilization, do not give any change on activities and spectral behavior.

Table II. *Effect of Mg^{2+} and Mn^{2+} on PSII Activity, Fluorescence Ratio, and Photoacoustic Ratio of Native and Immobilized Thylakoids*

	Ion Concn.	Activity	$\frac{F_{735}}{F_{695}}$	$\frac{PA_{676}}{PA_{440}}$
	mM	$\mu mol\ O_2\ mg^{-1}\ Chl\ h^{-1}$	ratio	
Mg^{2+}				
Native thylakoids	0	102	4.4	0.70
	1	208	2.6	0.75
	100	146	2	0.82
Immobilized thylakoids	0	56	3.7	0.72
	1	92	3	0.73
	100	30	2	0.75
Mn^{2+}				
Native thylakoids	0	240	4.2	0.73
	1	230	2.5	0.75
	100	26	1.7	
Immobilized thylakoids	0.01	100	3.2	0.76
	1	75	3	0.77
	100		1.8	

Furthermore, in the case of immobilized thylakoids the shapes of the 77 K fluorescence and PA spectra are not changed as compared to the native ones. Therefore, the observed losses of activities and the decreases in the F_{735} to F_{695} ratio after immobilization may be, at least partly, due to the barrier role (20) of the matrix (electron microscopy observations). When the optimal ion concentration (0.15 mM Na^+, 1 mM Mg^{2+}, 0.1 mM Mn^{2+}) is used with native and immobilized thylakoids, the increase of the PA_{676} to PA_{440} ratio is not significant (0.73 *versus* 0.75). In the presence of excess ion (except in the case of Mn^{2+}), the values are lower for immobilized thylakoids (0.75–0.76) than for native thylakoids (0.79–0.85). Even though PA data are not yet entirely understood (1), it seems that the low PA_{676} to PA_{440} ratio observed for immobilized thylakoids reflects an increase of the stability as compared to the native thylakoids.

Concerning the role played by Mn^{2+} (7), we have shown that its optimal concentration was 0.01 mM for native and 0.1 mM for immobilized thylakoids. In both cases, for concentrations greater than 1 mM, the PSI and PSII activities are highly inhibited, and especially the PA spectrum of immobilized thylakoids exhibits significant changes. Such modifications begin to appear with 100 mM Mg^{2+} (Fig. 7a) but remain unexplained.

In conclusion, the present work demonstrates the necessity to optimize the ionic content before the immobilization. The results point out the role played by the glutaraldehyde: inhibitor, stabilizing agent, and co-crosslinking agent. Due to little information (24) on the morphology of immobilized cells or organelles, and especially on the ultrastructural changes of immobilized thylakoids (9, 23), a functional, morphological characterization is required to determine the factors affecting their stability. In this way, the use of freeze fracture, which reveals the distribution of the particles located in stacked and unstacked membranes, should be of interest. Furthermore, measurements of the activities are not sufficient for a complete description of the immobilization effects. Methods providing information about energy transfer must be used in order to understand the modifications induced by the immobilization and also the interactions between the matrix and the biological material. Especially, fluorescence emission and PA spectroscopy have been used to correlate spectroscopic observations and kinetic measurements. We have shown no modification of the spectral shapes after the immobilization process and we can

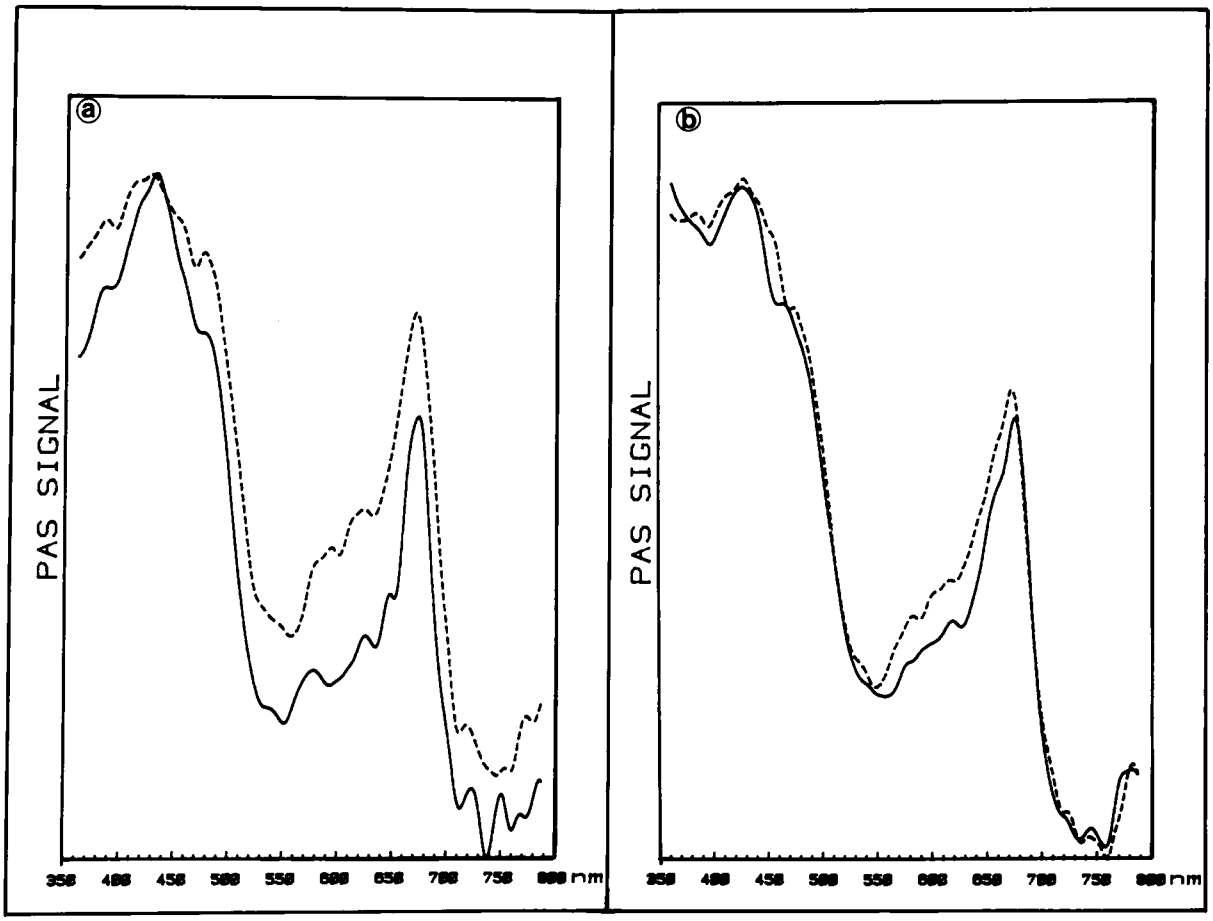

FIG. 7. PA spectra of native (a) and immobilized (b) thylakoids prepared in the absence (——) or in the presence (– – –) of 100 mM of Mg^{2+}.

explain changes in the peak ratios. In this way, the use of various fluorescence technique may complement such a study. The PA spectroscopy is of considerable interest in allowing direct studies on solid particles (which may be comprised of many photobiological immobilized systems such as algae, chromatophores. . .) and in spite of difficulties of interpretation, should be an attractive method for the future in this field.

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