On the Reconstitution of Photophosphorylation in CF_1-Extracted Chloroplasts

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(Z. Naturforsch. **31 c**, 445–451 [1976]; received March 15, 1976)

Photophosphorylation, Chloroplast Coupling Factor, Thylakoid Membrane Structure

1. A method for the reproducible preparation of CF_1-depleted thylakoid membranes was established. By repeated washes with pyrophosphate solution and subsequent treatment with sucrose media, containing micromolar concentrations of Ca^{2+} or Mg^{2+}, different degrees of CF_1 depletion and uncoupling were adjusted.

2. The membranes were completely uncoupled when about 50% CF_1 was removed. Re-coupling with isolated CF_1 was maximal at this point. It decreased with progressive CF_1 extraction.

3. The re-attached CF_1 particles regained their ability of energy-dependent adenine nucleotide exchange, an essential reaction in photophosphorylation.

4. CF_1-depleted membranes showed two main morphological alterations,
   a. unstacknig of the grana, and
   b. a perforation of the thylakoid membranes in those chloroplasts which showed a poor re-coupling activity. Whereas CF_1-depletion and unstacking were found to be reversible, the formation of holes was an irreversible effect.

5. The decreasing re-coupling activity was discussed in context to the irreversible perforation of the thylakoid membranes.

Introduction

Chloroplast coupling factor CF_1 has been discovered by Avron [1, 2], purified by Vambutas and Racker [3], and extensively studied by several workers [4–15]. Since its discovery it is well known that CF_1 can easily be removed from the thylakoid membranes by treatment with a chelating agent, and reattached to the membranes in the presence of excess metal ions. CF_1-depleted chloroplasts are uncoupled, in the reconstituted system photophosphorylation is reactivated [1–4, 15–17].

The reconstitution capacity of depleted membranes seems to depend on several factors which can not sufficiently be controlled. Thus the poor reproducibility of reconstitution experiments was complained [16, 18]. One critical point certainly is the state of preservation of the isolated enzyme. However, a more crucial problem is the preparation of CF_1-depleted membranes. It was reported that for optimum reconstitution of photophosphorylation CF_1 extraction must not exceed a critical percentage [19]. A reasonable explanation for this fact is still outstanding. Moreover, the suitable experimental conditions which guarantee the maintainance of that state of depletion, do not seem established.

A causal analysis of the varying reconstitution abilities of depleted membranes from lettuce chloroplasts was attempted by Shoshan and Shavit [15]. These authors reported that the decreasing re-coupling capacity was correlated with an increasing extraction of a protein which they believed to be an essential factor for coupling besides CF_1.

In the present paper a method for the reproducible preparation of CF_1-depleted thylakoid membranes from spinach chloroplasts is described. By this method a desired degree of CF_1-depletion, uncoupling, and re-coupling can be adjusted. Moreover the reasons for the varying reconstitution capacities were analyzed. The results indicate that with progressive CF_1 extraction an irreversible perforation of the thylakoid membranes occurs which prohibits the restoration of the primary energy conserving step.

Abbreviations: AdN, adenine nucleotide; CF_1, chloroplast coupling factor; Fecy, hexacyanoferrate (III); P_i, orthophosphate; Tricine, N-[tris (hydroxymethyl)-methyl] glycine.

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Methods

Spinach plants (*Spinacea oleracea*, var. "Vital") were either grown in a field, or in a greenhouse, or in a room under controlled conditions [20]. The origin

of the plant material did not affect the results of CF_1 depletion and reconstitution, although there were differences in the photochemical activities of the chloroplasts.

Chloroplasts were isolated from freshly harvested leaves as described in a previous paper [20]. The isolation medium contained 300 mM sucrose and 10 mM sodium pyrophosphate, pH 7.8. The chloroplasts were washed once with the same medium.

For preparation of CF_1-depleted membranes, the isolated chloroplasts were washed three times with 10 mM sodium pyrophosphate, pH 7.8. The pellets were then taken up in a medium which contained 300 mM sucrose and 2 mM Tris-Tricine buffer, pH 7.8 as the basic components. The latter step leads to CF_1 de-attachment [20]. By the addition of various concentrations of salts different degrees of CF_1-depletion were obtained (see Fig. 1).

CF_1 isolation was performed as reported elsewhere [20]. Purity of the preparations was controlled by disc-electrophoresis [21]. Usually it was better than 90%. The protein contents were determined by the method of Lowry *et al.* [22].

Electron transport and photophosphorylation were measured in a non-cyclic system with ferricyanide as electron acceptor. The incubation medium contained 25 mM Tricine buffer, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM ^{32}P-labelled orthophosphate, 1 mM ADP, 1 mM ferricyanide, and chloroplasts equivalent to about 10 μg chlorophyll/ml. The final volume was 2 ml, the temperature 20 °C, and the light intensity 1.5×10^6 ergs/cm^2 sec (white light). The incubation was carried out in small glas tubes which were inserted in a water bath.

Ferricyanide was determined photometrically by end point measurement [23], photophosphorylation was assayed by the incorporation of $^{32}P_i$ into the organic phosphate fraction [23]. Trypsin activation of membrane-bound Ca^{2+}-ATPase was performed as described before [20]. Ca^{2+}-ATPase was assayed at 36 °C [9]. The incubation contained 25 mM Tricine buffer, pH 8.0, 5 mM ATP, and 5 mM $CaCl_2$. The other conditions were the same as reported in a previous paper [20].

Negative staining of chloroplasts was performed with 2% phosphotungstic acid [24]. For sectioning preparation the chloroplasts were fixed by 2.5% (final concentration) glutaraldehyde [25]. After three washes with 100 mM phosphate buffer, pH 7.1, the pellets were treated with 1% OsO_4 (in 100 mM phosphate buffer, pH 7.1) [26]. Dehydratation was accomplished with increasing acetone concentrations and finally propylenoxide. The material was embedded in Durcopan ACM. After polymerization (3 days at 40 °C and 2 days at 60 °C) the chloroplasts were sectioned and contrasted with uranylacetate [27] and lead citrate [28].

Results

1. CF_1 depletion of thylakoid membranes

The CF_1-membrane bond is unspecifically mediated by metal ions [2]. Divalent as well as monovalent cations maintain the tight attachment of CF_1 particles on the thylakoid surface. Monovalent cations are required at much higher concentrations than divalent ones [2, 20]. Decrease of the cation concentration in a chloroplast suspension below a critical value induces de-attachment of CF_1. The degree of CF_1 depletion depends on the residual cation concentration if the other conditions are kept constant.

For cation extraction from chloroplast suspensions a two-step procedure was employed similar

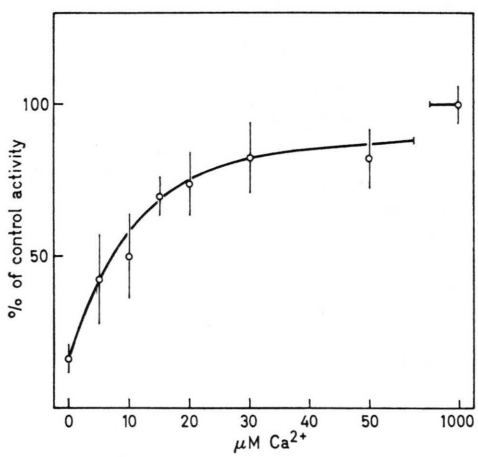

Fig. 1. Ca^{2+}-ATPase activity of pyrophosphate treated chloroplasts which were subsequently washed with sucrose media containing increasing concentrations of $CaCl_2$. The chloroplasts were twice washed with 10 mM sodium pyrophosphate, pH adjusted to 7.8. During washing a chlorophyll concentration of 30 μg/ml was kept. After centrifugation the supernatant was carefully discarded and the chloroplasts were resuspended in a medium which consisted of 0.3 M sucrose, 2 mM Tris-Tricine buffer, pH 7.8, and $CaCl_2$ at the concentrations indicated. The chlorophyll concentration was 10 μg/ml. After 20 min the chloroplasts were spun down and the pellets were re-suspended in a medium containing 0.3 M sucrose, 2 mM Tris-Tricine buffer, pH 7.8, and 5 mM $CaCl_2$. All steps were carried out at 4 °C. Ca^{2+}-ATPase activity was measured after trypsin activation of the membranes (see "Methods"). 3 independent experiments were performed, each with 2 parallel measurements. The values were related to the controls and averaged. The bars indicate the standard deviations. The mean Ca^{2+}-ATPase activities of the 3 controls were 190, 181, and 123 μmol P_i liberated/mg chlorophyll×hour.

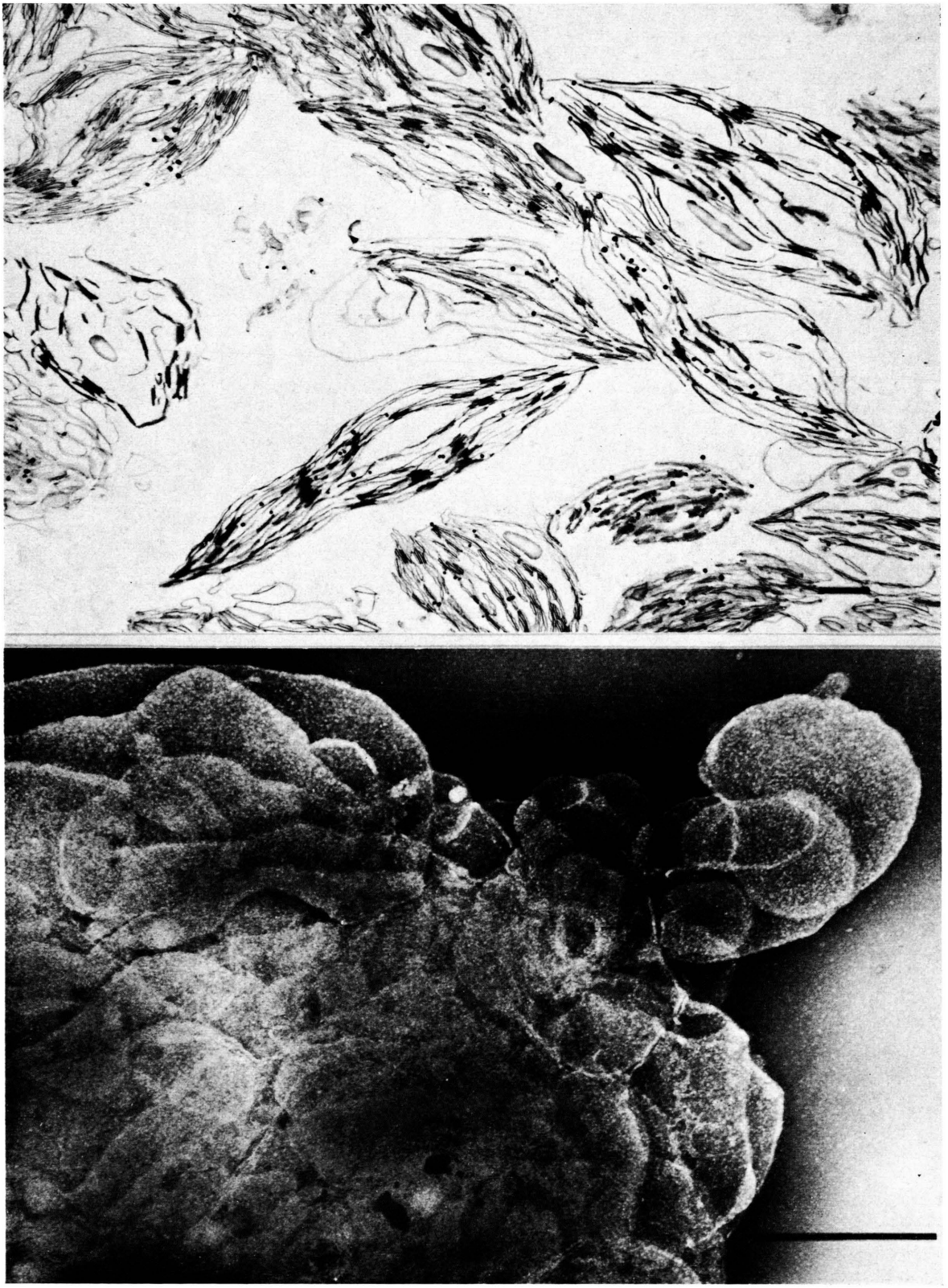

Fig. 3. Electron micrographs of chloroplasts treated with 1 mM $CaCl_2$ in the sucrose step. a. Thin section, b. negatively stained.

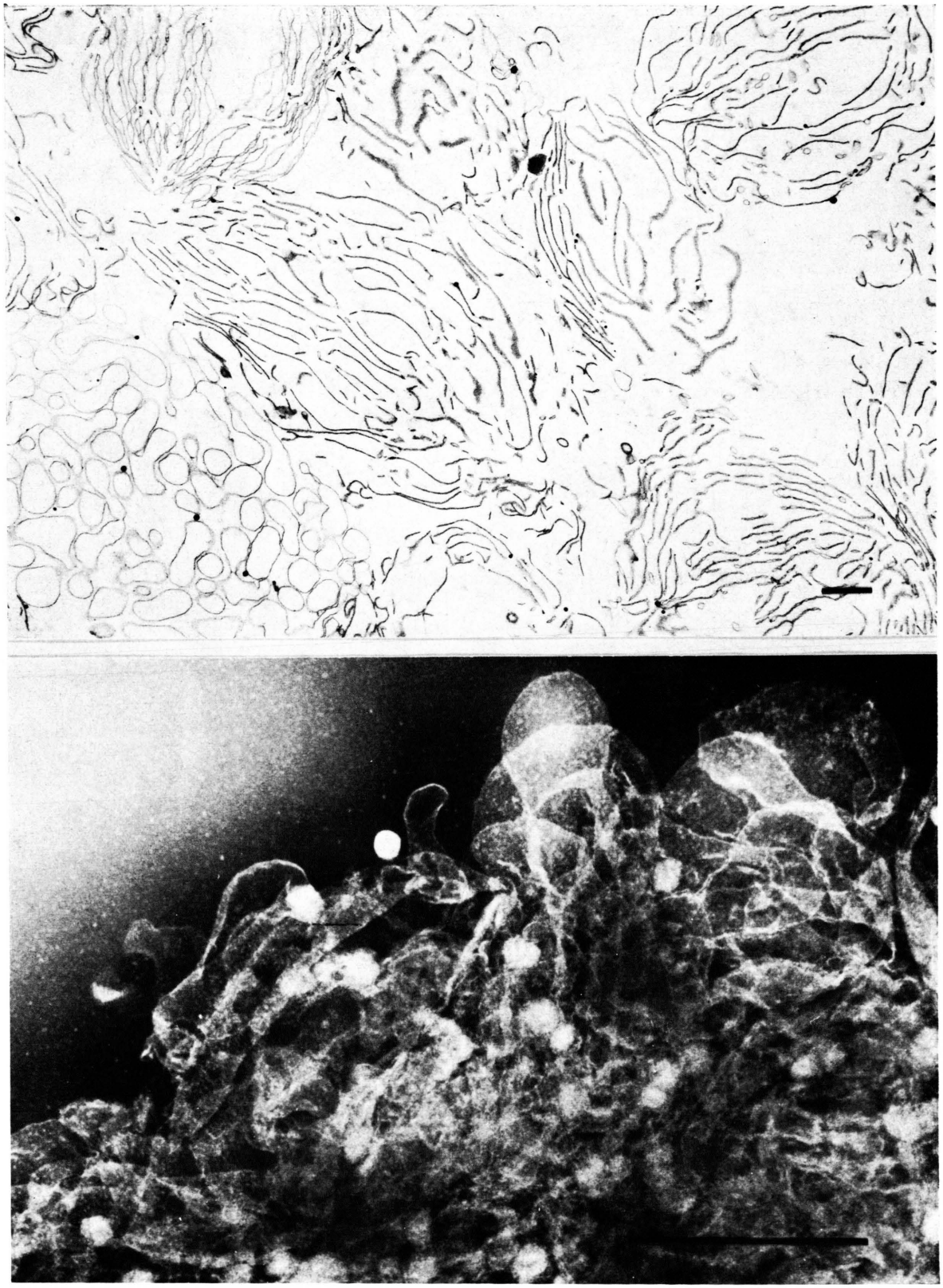

Fig. 4. Electron micrographs of chloroplasts treated with 11.5 μM $CaCl_2$ in the sucrose step. a. Thin section, b. negatively stained.

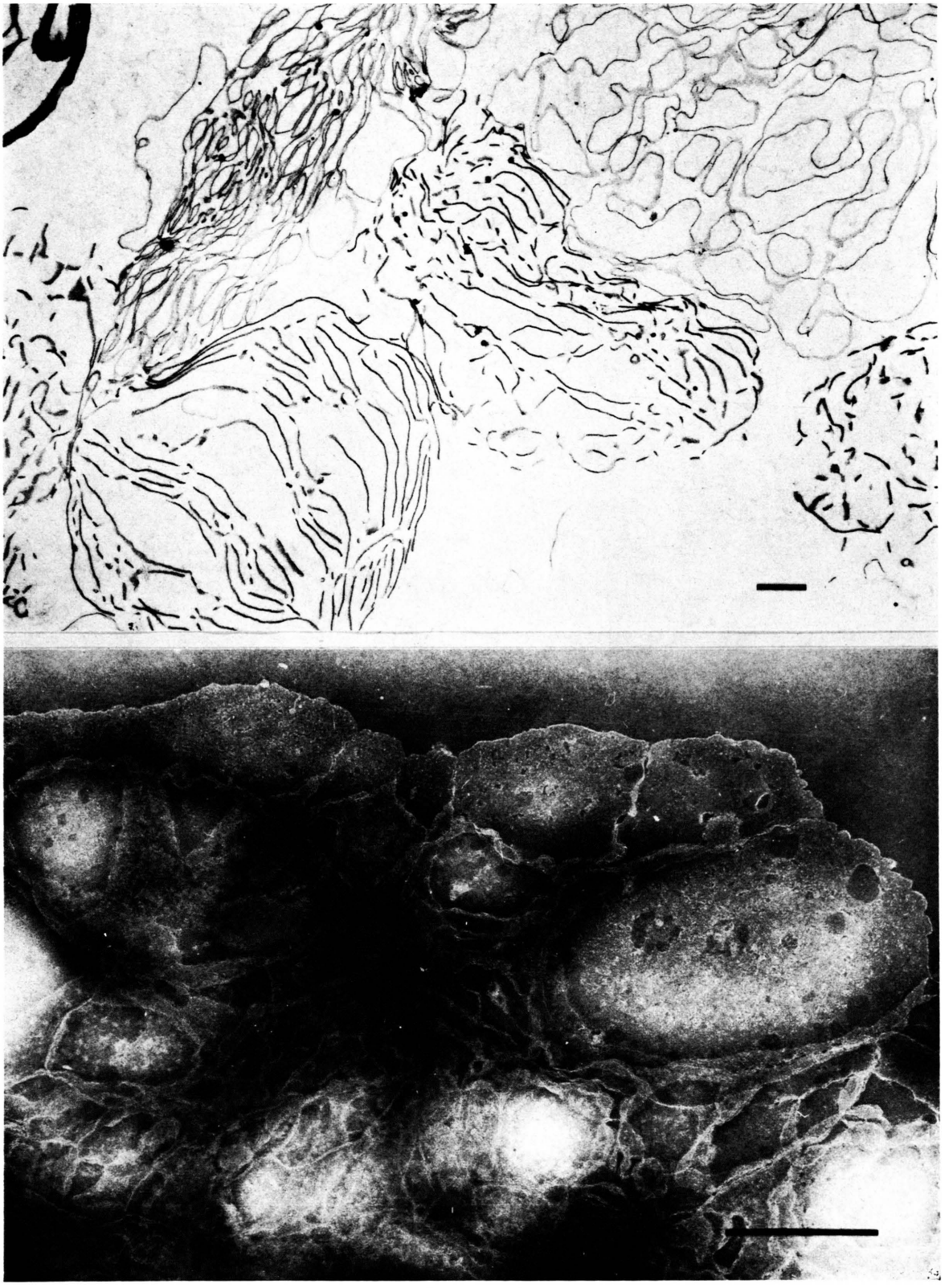

Fig. 5. Electron micrographs of chloroplasts treated with 6 μm $CaCl_2$ in the sucrose step. a. Thin section, b. negatively stained.

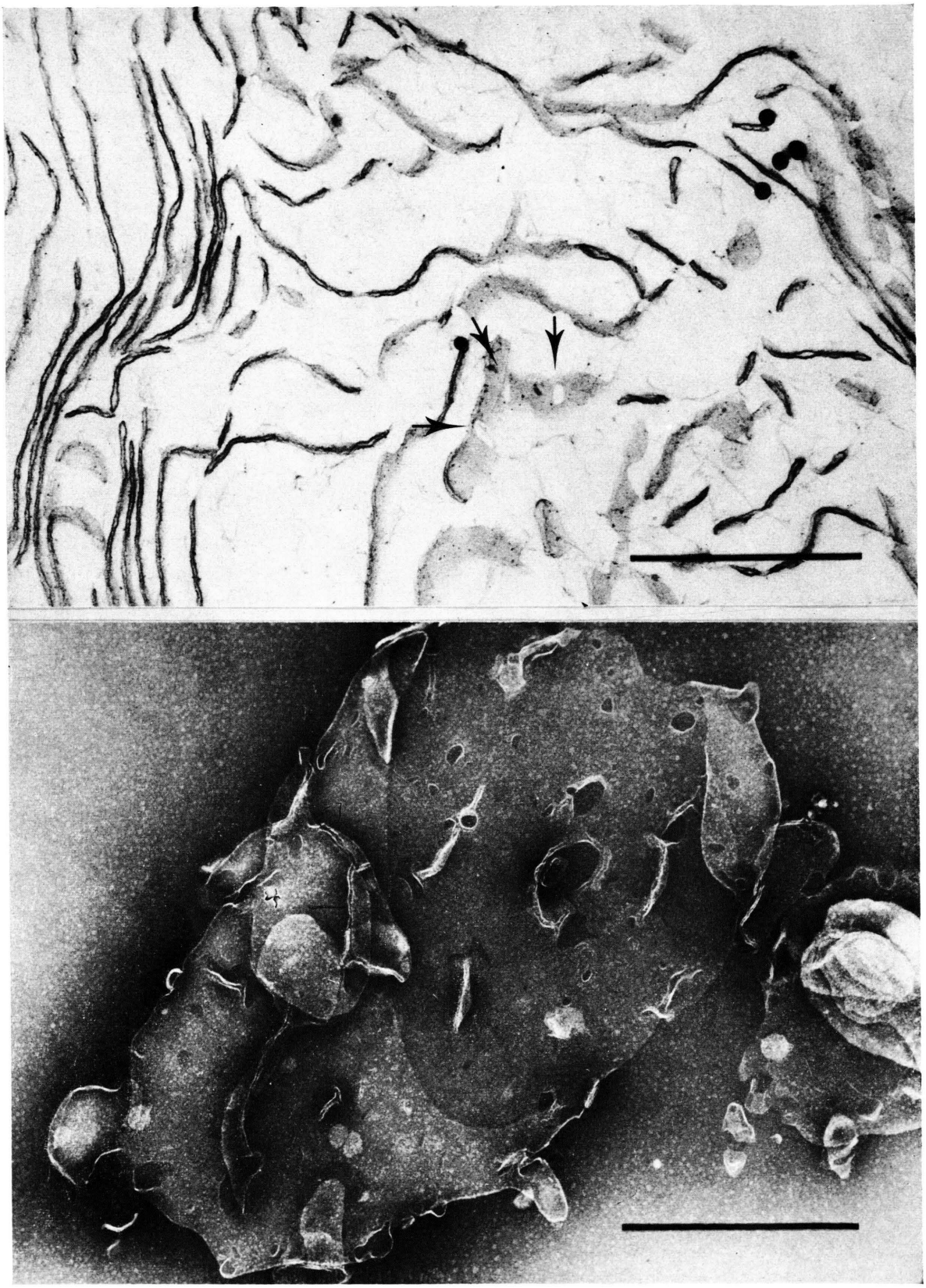

Fig. 6. Electron micrographs of chloroplasts treated with cation-free sucrose medium. a. Thin section; the arrows point to holes in the thylakoid membrane. b. Negatively stained.

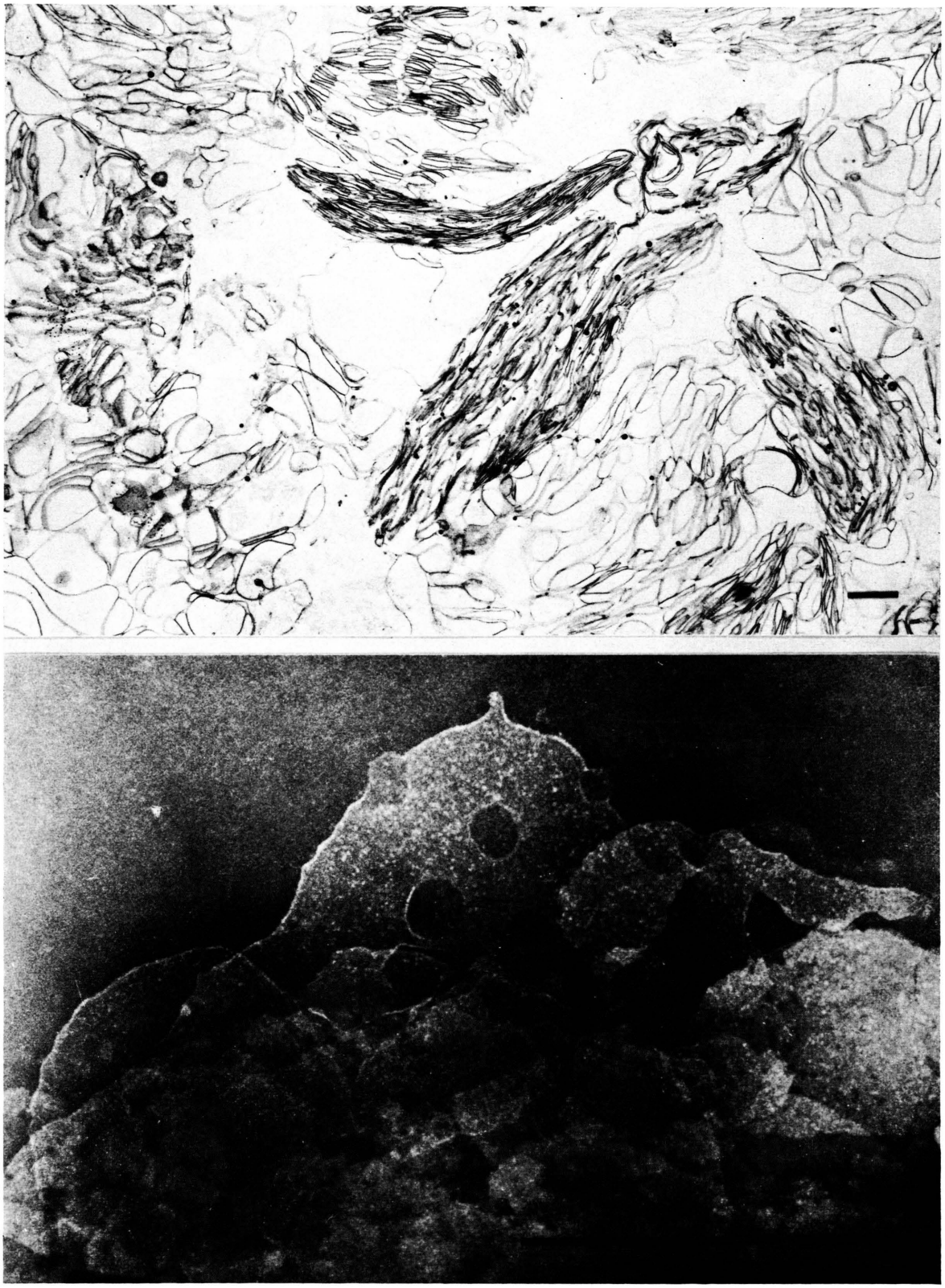

Fig. 7. Electron micrographs of chloroplasts which were treated with cation-free sucrose medium and subsequently recombined with CF_1 and 5 mM $CaCl_2$. a. Thin section, b. negatively stained.

to that which has been successfully used for CF_1 isolation [20]. In the first step for complexation of divalent cations pyrophosphate washes were performed. Three successive washes reduced the endogenous Mg^{2+} concentration down to almost zero. In the presence of 10 mM sodium pyrophosphate, which was used for the washes, the Na^+ concentration was sufficiently high for the maintainance of CF_1-membrane binding [20].

A subsequent treatment with a cation-free sucrose medium leads to a release of CF_1 [20]. If the medium additionally contained micromolar concentrations of Ca^{2+} or Mg^{2+}, CF_1 extraction was partially or totally prevented. At concentrations above 50 μM, virtually no ATPase activity could be detected in the supernatants. The contents of residual ATPase activity of the membranes as a function of Ca^{2+} concentration present in the sucrose step, follow a saturation curve (Fig. 1). 50% CF_1 extraction was attained at about 10 μM Ca^{2+}. With Mg^{2+} about 15 μM were required for the same effect. With Ca^{2+}- or Mg^{2+}-free sucrose medium, nearly 85% of total ATPase activity were extracted.

2. *Reconstitution of CF_1-extracted membranes with CF_1*

In the following experiments under the same conditions of CF_1 extraction, coupling was studied by measurement of the rates of electron transport and phosphorylation in a non-cyclic system. Furthermore the differentially depleted membranes were reconstituted with isolated CF_1. After recombination, coupling was again investigated. The results are shown in Fig. 2.

Three important results can be deduced from this experiment: a. Comparing Figs 1 and 2, a large deviation between the curves of coupling and CF_1 extraction is observed. The threshold Ca^{2+} concentration below which complete uncoupling appears, corresponds to the point of about 50% CF_1 depletion. That means coupling is much more sensitive towards cation shortage than CF_1 binding. b. The re-coupling capacity of the chloroplasts largely depends on the condition of the membranes before recombination with isolated CF_1. With increasing CF_1 depletion the re-coupling activity gradually decreases (see also in "Introduction"). c. The experiment permits the preparation of depleted membranes with an optimum re-coupling activity. Such membranes should be as much depleted and uncoupled as possible and recover a maximum degree of coupling upon reconstitution. Fully uncoupled membranes with a nearly 100% reactivation capacity are obtained if in the two-step extraction procedure the sucrose medium contains $10-12$ μM Ca^{2+}. This point is marked by the largest gap between the uncoupled and re-coupled curves in Fig. 2. Such membranes still contain about 50% of residual CF_1.

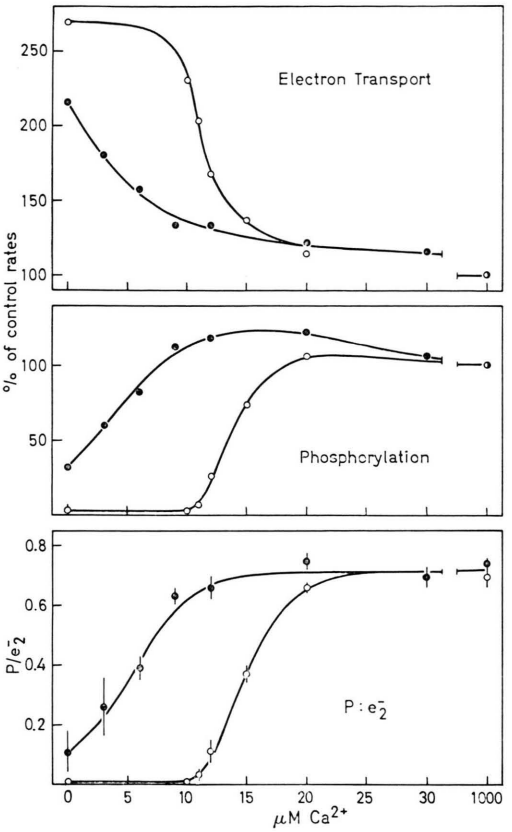

Fig. 2. Electron transport, photophosphorylation and $P:e_2^-$ of pyrophosphate treated chloroplasts which were subsequently washed with Ca^{2+} containing sucrose media, and of reconstituted chloroplasts. The chloroplasts were treated as described in Fig. 1, except that the final medium contained 5 mM $MgCl_2$ instead of $CaCl_2$. For reconstitution this medium additionally contained pure CF_1 at a concentration of 0.12 to 0.15 mg protein/ml. The final chlorophyll concentration varied between 0.22 and 0.26 mg/ml. Open symbols: pre-treated chloroplasts, closed symbols: reconstituted chloroplasts. 5 independent experiments were performed with 2 parallel measurements. The control rates of ferricyanide reduction varied between 268 and 518 μmol/mg chlorophyll$\times$hour; the phosphorylation rates ranged from 100 to 184 μmol/mg chlorophyll$\times$hour. The $P:e_2^-$ values were independently calculated for each experiment and averaged. The bars indicate the standard deviation. For electron transport and phosphorylation mean values related to the control rates were figured.

3. *Evidence for the functional integrity of re-attached CF_1*

Uncoupling of photophosphorylation by CF_1 extraction is referred to an increase of the proton permeability of the thylakoid membrane [4, 29–31]. In reconstituted membranes the formation of a light-dependent pH gradient is re-established [4]. That means CF_1 in some was closes the proton pores which have been opened by CF_1 removal. In this respect CF_1 can be replaced by N,N′-dicyclohexylcarbodiimide [30, 31]. Since a certain amount of residual CF_1 has to be present for re-coupling in our experiments, it is quite unsure whether the re-bound CF_1 particles recover their physiological function. If in the intact chloroplast CF_1 is not rate limiting for the overall process of photophosphorylation [19], the re-attached CF_1 particles might only passively stick the proton pores [31]. In order to judge about this possibility, a new experimental approach was attempted.

Table I. Energy-dependent adenine nucleotide exchange of CF_1, re-bound by depleted thylakoid membranes. For preparation of CF_1-depleted membranes the sucrose step contained 11.5 μM $CaCl_2$. [^{14}C]AdN-labelled CF_1 was prepared as described in a previous paper [33]. Thrice salt-washed broken chloroplasts were incubated in a medium consisting of 25 mM Tricine buffer, pH 7.8, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM methylviologen, and 22.5 μM [8-^{14}C]ADP. The chlorophyll content was 1 mg/ml. After 2 min illumination with white light (4.5×10^5 ergs/cm^2 sec) the chloroplasts were centrifuged and washed 3 times with a medium containing 25 mM Tricine buffer, pH 7.8 and 50 mM NaCl. The washed membranes were taken up in the same medium which additionally contained 5 mM EDTA. After centrifugation the pellet was re-suspended in 0.3 M sucrose + 2 mM Tricine buffer, pH 7.8. During this step labelled CF_1 was released into the supernatant. The supernatant was clarified by high speed centrifugation (150,000 × *g*, 1 hour). Purity of the preparation as checked by disc-electrophoresis was about 90%. The protein content was 0.138 mg/ml. Reconstitution of CF_1-depleted membranes with the labelled CF_1 was achieved as described under Fig. 2. In order to remove excess CF_1, after recombination the chloroplasts were washed with reconstitution medium. Release of CF_1-bound labelled adenine nucleotides was measured as described in a previous paper [33]. The incubation medium contained 25 mM Tricine buffer, pH 8.0, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM methylviologen, 0.1 mM ADP, and 1 mM P_i. The chlorophyll content was 0.193 mg/ml. The incubation time was 30 seconds, the light intensity (white light) 1.8×10^5 ergs/cm^2 sec. The incubation was performed at room temperature.

[^{14}C]AdN content of isolated CF_1	4.481 nmol/mg protein
[^{14}C]AdN content of reconstituted membranes	0.1822 nmol/mg Chl
Release of [^{14}C]AdN of reconstituted membranes	
dark	0.0207 nmol/mg Chl × 30 sec
light	0.0654 nmol/mg Chl × 30 sec

CF_1 contains firmly bound adenine nucleotides which can not be removed by washing [9, 12, 14, 32–34]. The bound adenine nucleotides only slowly exchange for added nucleotides unless CF_1 is transferred to another conformation by an energization of the thylakoid membrane [9, 32–34]. The energy-dependent adenylate exchange has to be regarded an essential step in the mechanism of photophosphorylation [32]. Thus, this reaction can be taken as an indicator of the functional integrity of a CF_1 molecule.

For a reconstitution experiment, CF_1 before isolation was labelled with [^{14}C]ADP in the light [33]. Subsequently the isolated labelled enzyme was re-attached to partially CF_1 depleted membranes and the exchange of bound adenylates was followed by measuring the release of label in the presence of unlabelled ADP. Table I shows that indeed an adenylate exchange in the re-bound CF_1 particles can be induced by light. This result indicates that in a reconstituted system the re-attached CF_1 molecules actually participate at least in part, in the process of photophosphorylation.

4. *Structural investigation of CF_1-extracted chloroplasts*

Chloroplasts which were treated in a way as described above (Figs 1 and 2), show two main morphological alterations as compared to control chloroplasts. One concerns the gross morphology of the thylakoid system, the other one the architecture of the thylakoid membrane itself. Both effects are induced by cation deficiency, however they seem to occur independently of each other at different degrees of cation shortage. The latter effect obviously impairs the re-coupling ability of the membranes.

In Figs 3 – 6 * electron micrographs of sectioned and negatively stained chloroplasts are shown. The chloroplasts after pyrophosphate washes were treated with sucrose media as described above. The sucrose media contained 1 mM Ca^{2+} (Fig. 3, control chloroplasts), 11.5 μM Ca^{2+} (Fig. 4, CF_1-depleted chloroplasts with an optimum re-coupling activity), 6 μM Ca^{2+} (Fig. 5, depleted chloroplasts with poor re-coupling activity) and no Ca^{2+} (Fig. 6, almost no re-coupling activity). In all chloroplasts except the controls, grana stacking is completely dissolved and the chloroplasts appear virtually agranal. The same effect has been observed by several authors [35–38]

* Figs 3 – 6 see Plates on pages 446 a – d.

under so-called "low salt conditions". Disorganization of the grana structure is reversible by re-addition of salts and it does not cause a loss of the photochemical activities [35, 36, 39]. Since salts of monovalent cations are required at higher concentrations than those of divalent cations, it is evident that the cation is the responsible ion species. Grana disorganization begins at cation concentrations well above those which induce CF_1 release [39].

A structural change of the thylakoid membrane itself is observed in chloroplasts which were treated with sucrose media containing less than 10 μM Ca^{2+} (Figs 5b and 6b). Such membranes after negative staining appear perforated. The formation of membrane holes seems to start at the peripheral thylakoids (Fig. 5b); upon treatment with Ca^{2+}-free medium completely disrupted membranes are observed (Fig. 6b). A preparation artifact (due to the rough technique of negative staining) may be excluded, since the holes can be observed even in the sectioned samples [+] (Fig. 6a).

Unlike grana disorganization and CF_1 extraction, membrane perforation due to cation shortage is an irreversible process. If sucrose treated membranes were reconstituted with CF_1 and excess Ca^{2+}, the membrane holes persisted although CF_1 particles were re-bound and a quasi-native grana structure was regained (Fig. 7 **). Under these conditions re-attachment of CF_1 can also be demonstrated by the biochemical assay. Table II shows that in the recombined membranes ATPase activity re-appeared although photophosphorylation remained completely uncoupled. However, the CF_1 occupation attained only 60% of the control value.

Table II. Coupling and Ca^{2+}-ATPase activity of extremely CF_1-depleted and reconstituted membranes. For CF_1 depletion cation-free sucrose medium was used in the final step. Reconstitution was performed as decribed in Fig. 2. The protein content of the CF_1 preparation was 0.135 mg/ml.

	μmol Fecy/mg Chl×h	μmol ATP/mg Chl×h	P/e_2^-	μmol P_i/mg Chl×h
Control	415	191	0.92	106
CF_1-depleted	972	1	0.00	9
1×reconstituted with CF_1	929	4	0.01	61
2×reconstituted with CF_1	891	4	0.01	61

[+] In sectioned chloroplasts the holes were first detected by Dr. S. Murakami in our laboratory.

** Fig. 7 see Plate on page 446 e.

Discussion

The equilibrium of CF_1-membrane binding seems to be affected by the cation concentration of a chloroplast suspension in a regular way. Accordingly different degrees of CF_1 depleted membranes can be prepared by adjustion of the cation concentration. This is accomplished by extraction of endogenous cations and re-addition of a fixed concentration of Ca^{2+} (or Mg^{2+}) ions.

Investigating the properties of differentially depleted membranes, besides CF_1 extraction morphological changes can be observed: unstacking of the grana and perforation of the thylakoid membranes. The three effects develop at different stages of cation shortage according to the sequence: disorganization of grana stacking – removal of CF_1 – membrane perforation. While the first two effects are reversible, membrane perforation is an irreversible process. It begins at a point where about 50% of CF_1 depletion is attained. From this point the re-coupling activity of the membranes gradually decreases. Thus the reconstitution capacity seems to be directly correlated with the degree of intactness of the thylakoid membranes. This can be reasonably interpreted in terms of the chemiosmotic hypothesis [40].

Concerning the proton permeability of extracted membranes two different effects have to be distinguished. Removal of CF_1 obviously leaves proton pores which prohibit the formation of an electron transport dependent pH gradient. These pores are sticked by CF_1 in the reconstituted system and the energy-dependent formation of a ΔpH or electric field, respectively, is re-established [4, 31]. By studying the energy-dependent exchange of CF_1-bound adenine nucleotides, we presented evidence that in the reconstituted membranes the re-attached CF_1-particles also recover their catalytic activity. Accordingly they seem to take their original morphological and functional position in the membrane.

Another effect is observed at more rigorous cation extraction. Under these condition the thylakoid membrane rips before all the CF_1 particles are removed. The membrane holes which arise from this event, are visible in the electron microscope. Under similar experimental conditions a membrane damage was suggested to occur by Girault *et al.* [16]. It seemed to be partially prevented by the addition of dithiothreitol. Since these holes are not closed by reconstitution, energy conservation by ΔpH formation remains impossible, although CF_1 is re-bound and

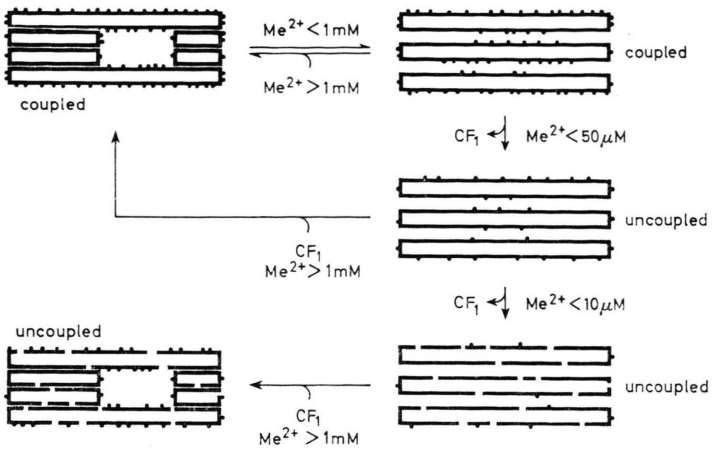

Fig. 8. Scheme of the effects induced by progressive cation shortage and by re-addition of cations and CF_1.

a grana structure is regained. A schematic presentation of the structural and functional effects caused by progressive cation extraction is given in Fig. 8.

The role of cations in grana stacking, CF_1-binding and the maintainance of the thylakoid membrane continuity is still unclear. Since all these effects are largely unspecific, we may suggest that the cations are required for compensation of negative surface charges. Accordingly electrostatic repulsive forces of the thylakoid surface would be reduced and the membranes are allowed to come together [23, 41, 42]. The actual binding forces which cause grana stacking, may be a combination of hydrophobic and electrostatic interactions [42]. A quite similar explanation might be considered for CF_1-membrane interaction. Moreover we may imagine that under certain conditions integral charged membrane constituents require the presence of counter-cations for the preservation of the continous membrane surface.

Financial support of the work by the Deutsche Forschungsgemeinschaft is acknowledged.

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