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EFFECTS OF VARIOUS CATIONS ON SEPARATION OF THE TWO PHOTOCHEMICAL SYSTEMS BY DIGITONIN TREATMENT

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SUMMARY

The effects of various cations were investigated during separation of the two photochemical systems of photosynthesis by digitonin treatment of spinach chloroplasts. Satisfactory separation was obtained only when an appropriate concentration of cations was present in the treatment medium; optimum concentrations for divalent cations (Mg^{2+}, Ca^{2+}, Ba^{2+}, Mn^{2+}) were 3–5 mM; for monovalent cations (Na^+, K^+, NH_4^+), 50 mM.

The modes of action of the salts (*e.g.* $MgCl_2$) were investigated. The presence of the cations in the digitonin treatment mixture was an essential factor for separation of the two photochemical systems. Similar effects of cations were also observed when chloroplasts were disrupted in a French pressure cell.

The effects of various salts and of light on the lamellar structure of the chloroplasts were investigated. There was no direct relationship between the electron microscopic changes of the chloroplast (*i.e.* presence or absence of stacking of lamellae) and the separation of the two photochemical systems in the digitonin treatment.

INTRODUCTION

Favorable effects of salts such as KCl or NaCl on the separation of Systems I and II particles of photosynthesis by detergent treatment (or mechanical disruption in a French pressure cell or in a sonic oscillator) have been reported by various investigators[1–3]. An effect of KCl was also noticed during our previous work aiming at the improvement of the separation of the two photochemical systems[4]. In subsequent experiments, it was discovered that addition of divalent ions, Mg^{2+}, Ca^{2+}, Ba^{2+} and Mn^{2+}, to the digitonin treatment medium was equally effective. The cationic nature of the salt effect was also indicated. This acticle describes the results of these experiments comparing the effects of monovalent and divalent cations.

On the other hand, ANDERSON AND VERNON[1] suggested that the preservation of the grana structure of the chloroplasts in the high-salt medium, which was found to be lost in the low-salt medium, was a necessary condition for the separation of the photochemical systems. Therefore, changes in the lamellar structure of chloroplasts induced by addition of various salts, or by illumination, were also investigated as to their effects on separation of the two systems by the digitonin treatment.

MATERIALS AND METHODS

Chloroplasts were isolated from spinach leaves, using a medium containing 0.05 M Tris–HCl buffer (pH 7.8) and 0.2 M NaCl. Digitonin treatment and subsequent sucrose density gradient centrifugation (51000 × *g* for 40 min; sucrose 15–55 % in 0.05 M Tricine buffer) were carried out as described previously[4]. In some experiments, chloroplasts (chlorophyll concentration of the suspension, 0.25 mg/ml) were disrupted in a French pressure cell at 300 kg/cm² for three successive times. A linear sucrose density gradient (15–45 % sucrose in 0.05 M Tricine buffer) was arranged in a centrifuge tube, containing a 1-ml layer of 55 % sucrose solution at the bottom.

Chlorophyll was determined by the method of ARNON[5]. The content of chlorophyll in each fraction of sucrose density gradient centrifugation was expressed as the absorbance at 678 nm. One unit of absorbance at 678 nm approximately corresponded to the chlorophyll concentration of 17 μg/ml. The contents of P700 were estimated by measuring the absorbance changes at 700 nm induced by oxidation–reduction; *i.e.* the absorbance difference, ascorbate *minus* ferricyanide. The photoreductions of $NADP^+$ and ferricyanide were measured spectrophotometrically by following the absorbance changes at 340 and 420 nm, respectively. The indicent intensity of actinic light was $5 \cdot 10^5$ erg/cm²·sec (> 600 nm).

The light-induced swelling of chloroplasts was observed in a medium containing 0.05 M Tris–HCl buffer (pH 7.8), 0.175 M NaCl, 0.01 M NH_4Cl and 20 μM phenazine methosulfate. After incubation in the dark for 15 min, chloroplast suspension (0.3 mg chlorophyll per ml, 8 ml), placed in a beaker with a flat bottom, was illuminated for 30 min at 20° at a light intensity of 30000 lux. The packed volume measurement was made by using hematocrit tubes. Centrifugation was carried out at 3000 × *g* for 20 min.

Electron microscopy was performed by the method described by NOBEL *et al.*[6]. Immediately after their incubation under various conditions, chloroplasts were treated with 1 % glutaraldehyde to stabilize their structure. The chloroplasts were then fixed successively in 5 % glutaraldehyde and 1 % OsO_4 in 0.05 M phosphate buffer (pH 6.5), each for 2 h, dehydrated in ethanol and embedded in Epon 812 resin. The ultrathin sections were examined under a Nihondenshi electron microscope (JEM-7A).

RESULTS AND DISCUSSION

Effects of $MgCl_2$ on the separation of the two photochemical systems by digitonin treatment

Fig. 1 shows the patterns of the linear sucrose density gradient centrifugation of chloroplast fragments obtained by digitonin treatment of chloroplasts in the presence of 5 mM $MgCl_2$. The whole green-colored material was distinctly separated into three bands, F-1, F-2 and F-3 (from top to bottom of the centrifugal tube). The molar ratios of chlorophylls *a* and *b* in the representative fractions for F-1, F-2 and F-3 were 5.0, 2.0 and 2.1, respectively. About 95 % of the P700 present in the digitonin-treated mixture was recovered in F-1. The Hill reaction activity with ferricyanide was exclusively localized in F-2 and F-3. These results indicate that an efficient separation of the two photochemical systems was obtained in the presence of 5 mM $MgCl_2$. The

results are in good agreement with those previously obtained with 0.05 M Tricine buffer containing 0.15 M KCl (ref. 4).

In order to examine the role of $MgCl_2$ on the separation of the two photochemical systems, the chlorophyll *a/b* ratios and Hill reaction activities were compared with the particle fractions obtained by digitonin treatment in the presence and absence of the salt (Table I). In this set of experiments, a rather low concentration of digitonin was used (0.4 % digitonin, 0.3 mg chlorophyll per ml) to lessen extensive disruption of the lamellae, which resulted in an inhibition of photochemical activities, especially in salt-free buffer. Table I shows that a significant separation of the two photochemical systems was obtained only when $MgCl_2$ was present in the digitonin treatment medium. In view of the values for chlorophyll *a/b* ratio (about 3.0) in p-4 and Sup. fractions obtained with the salt-free medium, the low levels of the Hill reaction activities in these fractions are believed to be due to their inactivation by an advanced disruption of the particles rather than an actual separation of the two photochemical systems. The presence of the Hill reaction activity with $NADP^+$ as electron acceptor in p-1 and p-2 in the $MgCl_2$-containing medium is due to an incomplete separation of the two photochemical systems with the concentration of digitonin used.

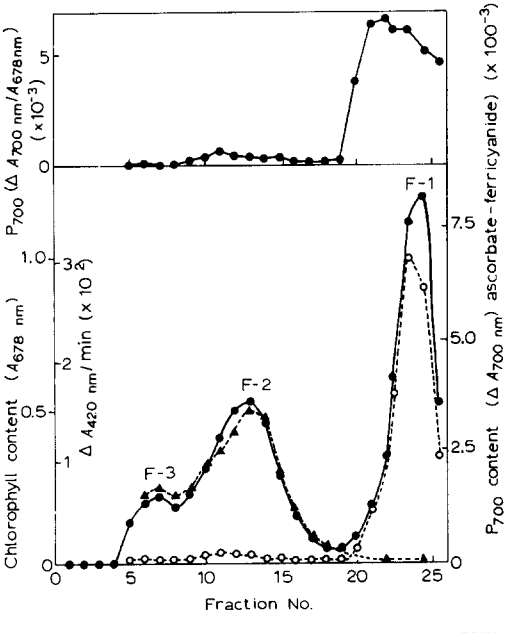

Fig. 1. Distributions of chlorophyll, P700 and Hill reaction activity (ferricyanide) in various fractions obtained by digitonin treatment in the presence of 5 mM $MgCl_2$. Digitonin incubation was carried out in 0.05 M Tricine buffer (pH 7.3) containing 5 mM $MgCl_2$ (digitonin, 0.5%; chlorophyll 0.3 mg/ml). The contents of chlorophyll and P700 are expressed as absorbance at 678 nm and the absorbance change at 700 nm induced by oxidation–reduction (difference, ascorbate *minus* ferricyanide). In the upper part of the figure are shown the values for P700/chlorophyll ($\Delta A_{700\ nm}/A_{678\ nm}$) in each fraction. Hill reaction activities with ferricyanide are expressed by $\Delta A_{420\ nm}$/min. The reaction mixture for the Hill reaction contained, in 1 ml: 50 μmoles of Tricine buffer, 10 μmoles of NaCl, 15 μmoles of methylamine–HCl, 0.4 μmoles of ferricyanide and particle fractions, 3–5 μg chlorophyll. Hill reaction activity (μmoles/mg chlorophyll per h): chloroplasts, 450; f-9, 113; f-13, 103. The total contents of chlorophyll in system I (F-1) and system II (F-2 and F-3) fractions were 51 and 49 %, respectively. ●—●, absorbance at 678 nm; ○---○, $\Delta A_{700\ nm}$; ▲---▲, $\Delta A_{420\ nm}$/min.

Fig. 2 shows the profiles of the sucrose density gradient centrifugation of the digitonin-treated mixture with and without the addition of $MgCl_2$. In the presence of 5 mM $MgCl_2$ (Fig. 2a), there was a separation of F-1 and F-2 particle fractions characterized with high and low P700 contents, respectively. The rather high content of P700 in F-3 in this experiment was due to an incomplete separation of the two photochemical systems with the low concentration of digitonin used. In contrast, in 0.05 M

TABLE I

CHLOROPHYLL CONTENTS AND HILL REACTION ACTIVITIES OF VARIOUS FRACTIONS OBTAINED BY DIGITONIN TREATMENT IN THE PRESENCE OR ABSENCE OF 5 mM $MgCl_2$

Chloroplasts isolated were washed once with 0.05 M Tricine buffer and suspended in the Tricine buffer with or without addition of 5 mM $MgCl_2$. Digitonin treatment: 0.4% digitonin; chlorophyll, 0.3 mg/ml. The digitonin-treated mixture was fractionated by differential centrifugation as shown in the table. Hill reaction activities are expressed in μmoles per mg chlorophyll per h.

Incubation medium	*0.05 M Tricine*			*0.05 M Tricine + 5 mM $MgCl_2$*		
	Chlorophyll a/b	*Hill reaction*		*Chlorophyll a/b*	*Hill reaction*	
Fractions		*$K_3Fe(CN)_6$*	*NADP+*		*$K_3Fe(CN)_6$*	*NADP+*
Digitonin-treated mixture	3.0	116	—	3.0	123	—
p-1, 1000 × *g*, 10 min	2.8	236	31	2.7	195	49
p-2, 10000 × *g*, 30 min	3.0	225	41	2.5	108	45
p-3, 50000 × *g*, 30 min	2.9	178	38	4.8	47	0
p-4, 144000 × *g*, 60 min	3.3	41	20	5.8	0	0
Sup.	2.8	21	0	4.8	0	0

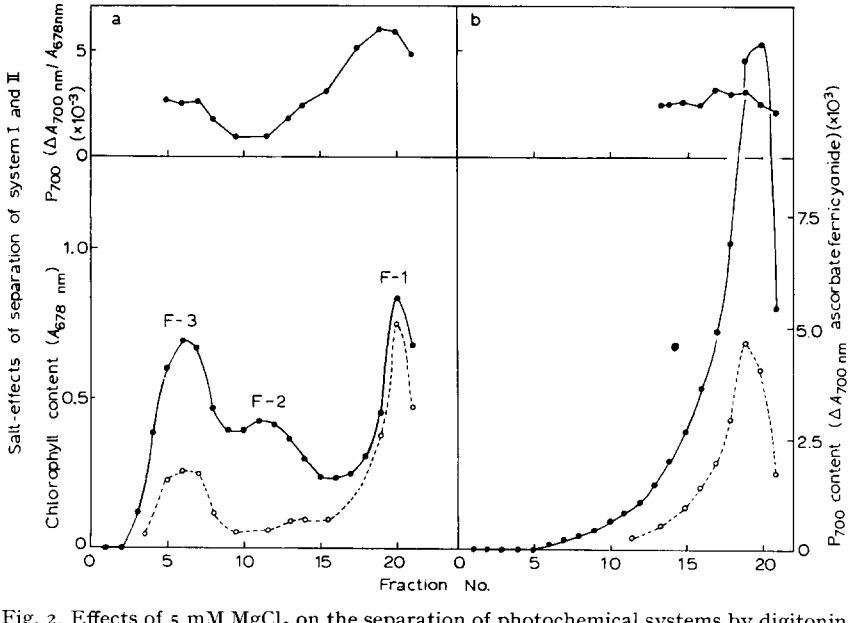

Fig. 2. Effects of 5 mM $MgCl_2$ on the separation of photochemical systems by digitonin treatment; patterns of the sucrose density gradient centrifugation. Conditions for digitonin treatment are the same as for Table I. a. In 0.05 M Tricine buffer containing 5 mM $MgCl_2$. b. In 0.05 M Tricine buffer. ●—●, absorbance at 678 nm; ○---○, $\Delta A_{700\ nm}$.

Tricine buffer without addition of $MgCl_2$ (Fig. 2b), most of the green material remained at the top of the centrifuge tube. P700 was evenly distributed among the fractions thus obtained.

The differences in modes of action of digitonin in the presence or absence of $MgCl_2$ were also observed in experiments in which a still lower concentration of digitonin (0.25 %) was used (Fig. 3). Comparison of the uppermost curves of Figs. 3a and 3b, representing the relative values for P700/chlorophyll in each fraction, shows that a significant separation of the two photochemical systems took place only when $MgCl_2$ was present in the digitonin treatment medium. In the experiment shown in Fig. 3b, centrifugation was continued for a longer period (80 min) to demonstrate that in the $MgCl_2$-free buffer, all the green material moved down essentially as one component: almost uniform distributions of P700 and Hill reaction activity throughout all the fractions will be noticed.

The modes of action of monovalent and divalent cations

The optimum concentration of $MgCl_2$ for the separation of photochemical systems was 3–5 mM. At higher concentrations of $MgCl_2$(10 mM), there was an increased tendency for aggregation of the green material in the digitonin-treated mixture; at lower concentrations, *e.g.* 1 mM the separation of the two photochemical systems was incomplete.

When 10 mM EDTA was added before digitonin treatment in Tricine buffer containing $MgCl_2$, the subsequent density gradient centrifugation gave the same dis-

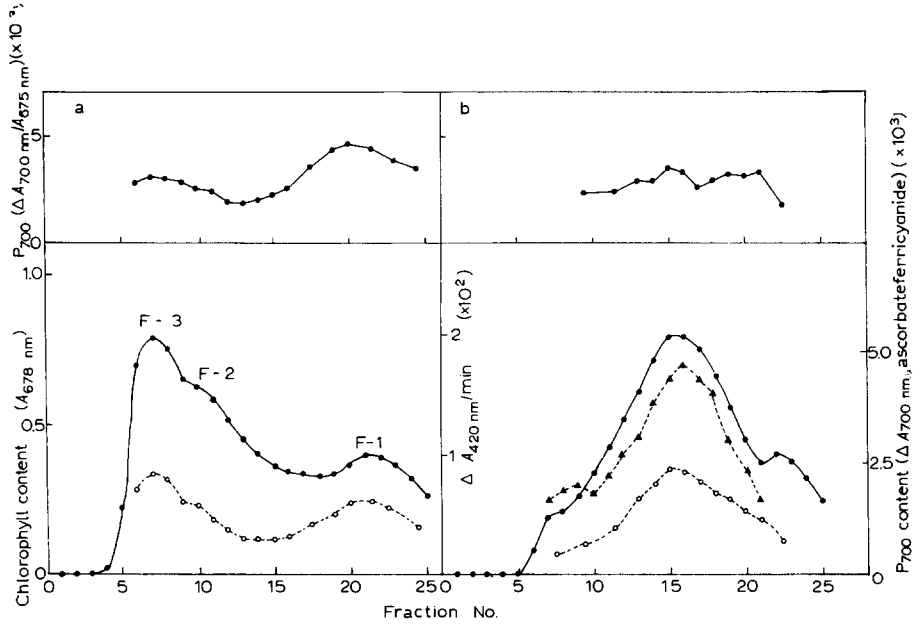

Fig. 3. Patterns of sucrose density gradient centrifugation of chloroplasts treated with a low concentration of digitonin. Digitonin treatment, 0.25% digitonin; chlorophyll, 0.3 mg/ml. a. In 0.05 M Tricine buffer containing 5 mM $MgCl_2$. Centrifugation was carried out under standard conditions, *i.e.* 51000 × *g* for 40 min. b. In 0.05 M Tricine buffer. Centrifugation was carried out at 51000 × *g* for 80 min. Hill reaction activity with ferricyanide (μmoles/mg chlorophyll per h); chloroplasts, 215; f-8, 105; f-15, 75. ●---●, absorbance at 678 nm; ○---○, $\Delta A_{700\ nm}$; ▲---▲ $\Delta A_{420\ nm}$/min.

tribution pattern as that obtained with the Tricine buffer without addition of the salt. This finding indicates that the observed effect of $MgCl_2$ was due to the presence in the medium of the cation, Mg^{2+}, which was captured by the chelating reagent.

Effects of divalent cations other than Mg^{2+} were tested. Ba^{2+}, Mn^{2+} and Ca^{2+} were also effective for separation of the photochemical systems over the same concentration range as for $MgCl_2$ (5 mM).

Monovalent cations, including Na^+, K^+ and NH_4^+ (as chloride salts), were equally effective for separation of the two photochemical systems. The necessary concentration with these salts was about 50 mM; 10 mM was too low; 50–150 mM was the adequate concentration range. ANDERSON AND VERNON[1], who investigated the effects of KCl and some ionic buffers, suggested that the level of ionic strength of the medium is the main factor affecting the separation of the photochemical systems. However, the observed difference in levels of optimum concentrations for the monovalent and divalent cations cannot be explained in terms of the difference in the ionic strength of the medium, but there are specific effects depending on the nature of the ions; in favor of the divalent cations as compared with the monovalent cations examined.

In the experiment in which chloroplasts were disrupted in a French pressure cell, similar circumstances were encountered on the effects of monovalent and divalent cations for separation of the two photochemical systems. The necessary concentrations were also 3–5 mM for divalent cations and 50 mM for monovalent cations.

The following experiments were carried out to test whether previous contact of chloroplasts with 5 mM $MgCl_2$ determines, in an irreversible way, the separation of the two photochemical systems in the subsequent digitonin treatment. Chloroplasts, isolated in a medium containing 0.05 M Tris–HCl buffer (pH 7.8) and 0.2 M NaCl, were suspended in 0.05 M Tricine buffer containing 5 mM $MgCl_2$ (pre-treatment medium). After standing for 15 min at 0°, the chloroplasts were subjected to digitonin treatment in the Tricine buffer, with and without addition of $MgCl_2$, and tested for separation of the photochemical systems. Another set of experiments was carried out in a similar way, but $MgCl_2$ was omitted from the pre-treatment medium. In the experimental results with respect to the patterns of sucrose density gradient centrifugation, the contents of P700 and chlorophyll *a*/*b* ratios indicated that the presence of $MgCl_2$ in the medium for digitonin treatment was essential for the separation of the two photochemical systems, irrespective of the presence or absence of $MgCl_2$ in the pre-treatment medium.

When $MgCl_2$ was removed from the mixture by passing it through a Sephadex column after the digitonin treatment, or when $MgCl_2$ (5 mM) was added to the mixture after digitonin treatment in the $MgCl_2$-free buffer, the sucrose density gradient centrifugation in both cases gave rise to similar patterns of particle distribution as compared with those of the control experiments, without gel filtration or without later addition of $MgCl_2$. These results also indicate that the presence of $MgCl_2$ during digitonin treatment was essential for separation of the two photochemical systems.

Relationship between changes in chloroplast structure and the separation of the two photochemical systems by digitonin treatment

IZAWA AND GOOD[7] studied the electron microscopic structure of chloroplasts suspended in Tricine buffer and observed striking changes induced by addition of various salts. As reported by IZAWA AND GOOD[7], in the 0.05 M Tricine buffer containing

0.2 M sucrose, chloroplasts appeared as an ensemble of fully extended sheets of closely paired membranes; showing no grana stack (Fig. 4a). When 5 mM $MgCl_2$ was added to such a chloroplast suspension, some parts of the lamellae stuck together to reconstitute a structure reminiscent of the regular grana stacks in the original chloroplasts (Fig. 4b). When sucrose was omitted from the above medium, the lamellae underwent a more extensive swelling owing to the hypotonic condition of the medium (Fig. 4c). On adding 5 mM $MgCl_2$ to such lamellae, close stacking took place between parallel sheets of lamellae, although the structure thus formed was rather removed from that of the

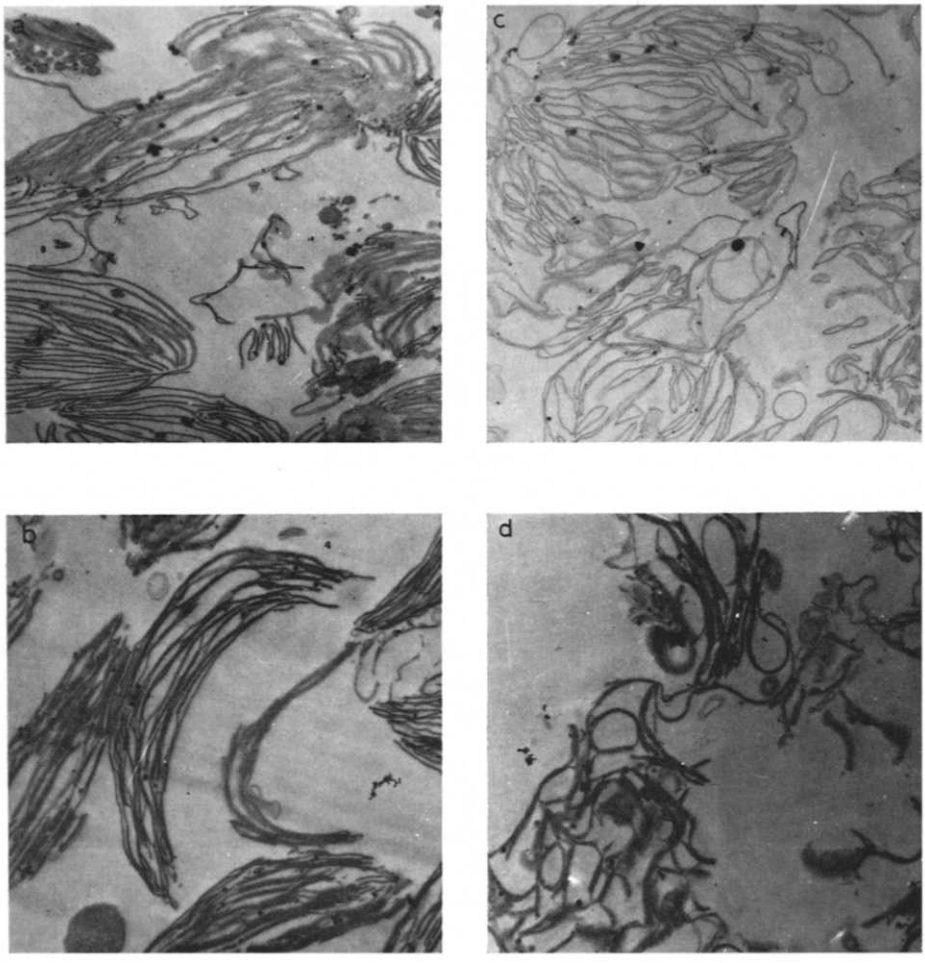

Fig. 4. Electron micrographs showing structural changes induced by addition of $MgCl_2$ to chloroplasts suspended in salt-free Tricine buffer. Chloroplasts isolated in 0.05 M Tris–HCl buffer containing 0.2 M NaCl were suspended in 0.05 M Tricine buffer or in the same buffer containing 0.2 M sucrose. After 15 min, 5 mM $MgCl_2$ was added. After another 15 min, glutaraldehyde was added (final concentration, 1%). The chloroplasts were collected by centrifugation and were subjected to fixation with 5% glutaraldehyde and 1% OsO_4 in 0.05 M phosphate buffer (pH 6.5). Magnification, 6750 ×. (a) Sucrose + Tricine buffer. (b) Sucrose + Tricine buffer + 5 mM $MgCl_2$. (c) Tricine buffer. (d) Tricine buffer + 5 mM $MgCl_2$.

regular grana stacks in the original chloroplasts (Fig. 4d)*. Similar structural changes were observed when 100 mM NaCl was added instead of $MgCl_2$. The concentration ranges of monovalent and divalent cations required for these structural changes were almost the same as those needed for separation of the two photochemical systems in digitonin treatment. These findings seemed to be in harmony with the suggestion made by ANDERSON AND VERNON[1] that the presence or absence of the grana structure of the chloroplast lamellae, according to the presence or absence of salts in the medium, is directly connected with the capacity for separation of the two photochemical systems in the digitonin treatment. However, the following facts are clearly in disagreement with their view.

As reported by CROFTS *et al.*[8], chloroplasts suspended in 0.1 M NH_4Cl solution showed a considerable swelling of the lamellae owing to the hypotonic condition of the medium, but some parts of the grana stacking still remained preserved (Fig. 5a). In contrast, in 0.1 M ammonium acetate solution, there was a much more extensive swelling of lamellae, resulting in an almost entire loss of the regular pattern of lamellar structure (Fig. 5b). The digitonin treatment, in the presence of these salts in the medium resulted in an equally efficient separation of the photochemical systems as judged by the distributions of chlorophyll and P700 in the fractions obtained by the subsequent centrifugation on a linear sucrose density gradient. Washing of the salt-treated chloroplasts with salt-free Tricine buffer also resulted in the failure to separate the photochemical systems by subsequent digitonin treatment in the salt-free medium.

Another set of experiments was carried out with chloroplasts whose structure had been profoundly altered by illumination. When chloroplasts suspended in 0.05 M Tris–HCl buffer containing 0.175 M NaCl, 0.01 M NH_4Cl and 20 μM phenazine methosulfate were illuminated, there occurred an extensive swelling of the lamellae; the regular lamellar structure of chloroplasts was found to be completely disrupted; there was no grana stack, the electron micrographs showing only a swarm of swollen vesicles (Fig. 6b). The packed volume of the illuminated chloroplasts was about 6 times as large as that in the dark. The digitonin treatment of such light-swollen chloroplasts in the same medium gave rise to essentially similar results as with the non-illuminated control; namely, a satisfactory separation of the two photochemical systems was obtained with both materials.

These findings indicate that the separation of the two photochemical systems can also occur, after swelling or extensive disruption of the lamellae, only if appropriate concentrations of cations are present in the medium for digitonin treatment. It seems that the structural changes reflected by different appearances in the electron micrographs in the presence and absence of various cations are not directly correlated with the differences in the state of the lamellae which affect the capacity for separation of the two photochemical systems. Although a definite statement on the mechanisms of these effects of cations cannot be drawn from the results of the present study, it is inferred that the cations in some way weaken the binding between Systems I and II particle units in the lamellae of chloroplasts eventually to facilitate separation of the two photochemical systems by the detergent treatment.

Of interest in connection with the above-described observations are the recent

* After S. MURAKAMI (private communication, unpublished data), a detachment of lamellar stacking in the salt-free medium and its recovery on addition of salts are also demonstrated when deionized water was used instead of Tricine buffer as in the present study.

findings of MURATA[9] and MURATA *et al.*[10] that various cations, in the same concentration range as was effective for separation of photochemical systems in the present experiments, altered the distribution of excitation energy between the two pigment systems of photosynthesis. On a basis of analyses of the chlorophyll *a* fluorescence in the chloroplasts, these authors concluded that the addition of cations suppresses the transfer (overspill) of the excitation energy from System II to System I.

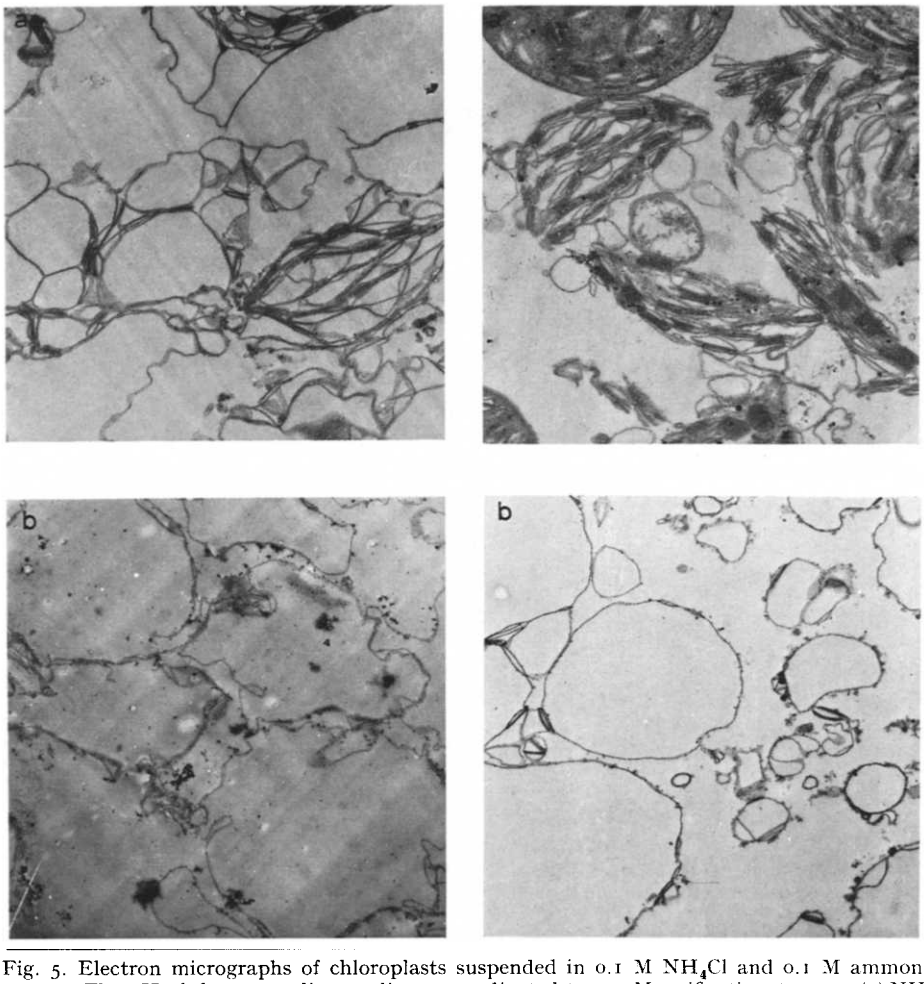

Fig. 5. Electron micrographs of chloroplasts suspended in 0.1 M NH_4Cl and 0.1 M ammonium acetate. The pH of the suspending medium was adjusted to 7.4. Magnification, 6750 ×. (a) NH_4Cl. (b) Ammonium acetate.

Fig. 6. Electron micrographs showing extensive swelling of chloroplasts by illumination. The reaction medium contained 0.05 M Tris–HCl buffer pH(7.8), 0.175 M NaCl, 0.01 M NH_4Cl and 20 μM phenazine methosulfate. Magnification, 6750 ×. (a) Dark. (b) Illuminated.

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