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EFFECTS OF CATIONS UPON CHLOROPLAST MEMBRANE SUBUNIT INTERACTIONS AND EXCITATION ENERGY DISTRIBUTION

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SUMMARY

When isolated chloroplasts from mature pea (*Pisum sativum*) leaves were treated with digitonin under "low salt" conditions, the membranes were extensively solubilized into small subunits (as evidenced by analysis with small pore ultrafilters). From this solubilized preparation, a photochemically inactive chlorophyll · protein complex (chlorophyll *a*/*b* ratio, 1.3) was isolated. We suggest that the detergent-derived membrane fragment from mature membranes is a structural complex within the membrane which contains the light-harvesting chlorophyll *a*/*b* protein and which acts as a light-harvesting antenna primarily for Photosystem II.

Cations dramatically alter the structural interaction of the light-harvesting complex with the photochemically active system II complex. This interaction has been measured by determining the amount of protein-bound chlorophyll *b* and Photosystem II activity which can be released into dispersed subunits by digitonin treatment of chloroplast lamellae. When cations are present to cause interaction between the Photosystem II complex and the light-harvesting pigment · protein, the combined complexes pellet as a "heavy" membranous fraction during differential centrifugation of detergent treated lamellae. In the absence of cations, the two complexes dissociate and can be isolated in a "light" submembrane preparation from which the light-harvesting complex can be purified by sucrose gradient centrifugation.

Cation effects on excitation energy distribution between Photosystems I and II have been monitored by following Photosystem II fluorescence changes under chloroplast incubation conditions identical to those used for detergent treatment (with the exception of chlorophyll concentration differences and omission of detergents). The cation dependency of the pigment · protein complex and Photosystem II reaction center interactions measured by detergent fractionation, and regulation of excitation energy distribution as measured by fluorescence changes, were identical. We conclude that changes in substructural organization of intact membranes, involving cation

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Abbreviations: DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea (Diuron); DCPIP: dichlorophenol indophenol; SDS: sodium dodecylsulfate.

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induced changes in the interaction of intramembranous subunits, are the primary factors regulating the distribution of excitation energy between Photosystems II and I.

INTRODUCTION

Light-induced electron transport in chloroplasts is thought to involve two photosystems acting in series. For optimal quantum efficiency of the non-cyclic electron transport pathway, absorbed radiant energy must be equally partitioned between each of the two photochemical reaction centers [1]. It is now established that cations in some way regulate the distribution of absorbed excitation energy between Photosystems I and II in isolated chloroplasts [2, 3]. The mechanism by which this regulation occurs is not known.

It has recently been demonstrated that there is a parallel pattern of appearance of a light-harvesting chlorophyll *a*/*b* protein and the ability of cations to regulate exciton transfer between the two photosystems in greening chloroplast membranes [4, 5]. It was suggested that this pigment · protein is intimately involved in the cation-mediated regulatory process. The mechanism by which the pigment · protein could be exerting its effect has not been elucidated, however, although structural studies of the greening chloroplast membranes did indicate changes in membrane organization might be involved. Evaluation of these aspects of the problem requires a means of analyzing membrane substructure.

Conceptual interpretations of chloroplast membrane substructure have been greatly influenced by membrane fractionation experiments. Boardman and Anderson [6, 7] and Vernon and coworkers [8] have demonstrated that detergent treatment of isolated chloroplast lamellae followed by differential centrifugation results in recovery of two distinctly different types of submembrane fragments: a heavy fraction recovered after low speed centrifugation which has a low chlorophyll *a*/*b* ratio and which was enriched in Photosystem II activity, and a light fraction which has a high chlorophyll *a*/*b* ratio and only Photosystem I activity.

One interesting anomaly has been demonstrated in certain fractionation experiments. If chloroplasts were suspended in low ionic strength media during detergent treatment, Photosystems I and II could not be separated by differential centrifugation. In contrast to the above-mentioned studies in which Photosystem II sedimented after low speed centrifugation, Photosystem II activity was found in both light and heavy fractions following low-salt detergent treatment [9]. The reason for the conversion of the Photosystem II complex from a heavy to light form was not understood.

The generally accepted concept that chloroplast membranes contain two types of subunits has been amended in some recent studies. Wessels and coworkers [11, 12] have found that three different kinds of chlorophyll-containing particles can be obtained from digitonin-treated lamellae: two different photochemically active particles that represent Photosystems I and II and a third photochemically inactive complex with a very low chlorophyll *a*/*b* ratio were recovered from sucrose gradients of digitonin-solubilized membranes. Vernon and Klein [13] have also suggested that there are three structural units within chloroplast lamellae based on more indirect observations during their fractionation studies using Triton X-100. In all of these

latter experiments, it has been shown that both the Photosystem I and Photosystem II complexes have high chlorophyll *a*/*b* ratios, whereas chlorophyll *b* is associated with photochemically inactive material.

MATERIALS AND METHODS

Pea (*Pisum sativum* var. Laxton Progress) seedlings were grown under cool-white fluorescent light (16 h photoperiod) in vermiculite moistened with ½ strength Hoagland's solution. Washed pea leaves (50 g) were ground for 15 s in a Waring Blender in 100 ml of 0.1 M Na-Tricine (pH 7.8) containing 0.4 M Sorbitol. The resulting brei was filtered through 4 and then 12 layers of cheesecloth. A chloroplast pellet was obtained by centrifugation at $1000 \times g$ for 10 min. The pellet was thoroughly dispersed in 30 ml of 0.2 M sucrose and then centrifuged at $2000 \times g$ for 10 min. This sucrose wash was repeated once. The resulting pellet was resuspended in 10 mM NaCl and 1 mM Na-Tricine, pH 7.8, plus various amounts of KCl or $MgCl_2$ as described for each experiment.

"Intermittent light plastids" were prepared from pea seedlings which were grown for 7 days in darkness and then for 48 h in cycles of intermittent light (2 min light, 2 h dark) as previously described [4]. The conditions for chloroplast isolation were as described above.

For detergent incubation experiments, the washed, resuspended chloroplast pellet described above was diluted to 0.5–1.0 mg chl/ml in 10 mM NaCl, 1 mM Na-Tricine, pH 7.8, plus varying amounts of either $MgCl_2$ or KCl. Recrystallized digitonin was then added to give 10 mg detergent/ml. The mixture was incubated with stirring for 30 min at 20 °C. The solution was subsequently centrifuged at $40\,000 \times g$ for 30 min to generate a heavy pellet and a light supernatant. In some experiments the light material was pelleted by centrifugation at $144\,000 \times g$ for 1 h.

For sucrose gradient separation of submembrane fragments, a linear gradient (1.0–0.1 M sucrose) was prepared. 5-ml of the light $40\,000 \times g$ supernatant fraction described above was layered on each gradient. The sample was then centrifuged for 16 h in a SW-27 rotor at $100\,000 \times g$. Samples were collected from each tube by forcing the sucrose out of the top of the gradient using a special tube-capping device. The eluant solution was scanned at 678 nm in a flow-through spectrophotometric cell and equal volume fractions were collected.

Photosystem II activity of samples was determined by monitoring the rate of DCPIP reduction in a Coleman 124 double-beam spectrophotometer modified for direct sample illumination. The reaction mixture contained 50 mM Na phosphate (pH 6.7), 5 mM NH_4Cl, 0.04 mM DCPIP, 1.25 mM DPC as electron donor (unless otherwise indicated) and 20–25 μg chl in a 2 ml reaction mixture.

In system I assays, methyl viologen was used as an electron acceptor and reduced plastocyanin as an electron donor. Reaction mixtures contained 50 mM Na phosphate (pH 6.7), 5 mM NH_4Cl, 1.3 mM sodium ascorbate, 0.02 mM DCMU, 0.8 mM methyl viologen and 0.8 mM NaN_3, saturating amounts of plastocyanin (purified by Anderson and McCarty's method) and 10–15 μg chlorophyll in a 1.5 ml volume. Auto-oxidation of methyl viologen was monitored by measuring O_2 consumption with a YSI Clark electrode in a temperature regulated reaction cell at 20 °C. A yellow Corning filter (No. 3-68) was placed between the light source and sample to

eliminate artifactual responses in the O_2 measurements. Fluorescence measurements were with an Aminco-Bowman spectrofluorometer using an S-20 phototube.

For electrophoretic separation of pigment protein complexes, samples were preincubated for 1 h in 0.1 M Tris buffer (pH 8.2) containing 1 % SDS and then subjected to electrophoresis on polyacrylamide gels containing 0.1 % SDS according to procedures previously described [5]. For separation of proteins from lipid extracted membranes, sample preparation and electrophoretic procedures of Hoober [15] were followed. Gels were scanned with a model 2410 linear transport mechanism fitted to a Gilford Model 240 spectrophotometer.

RESULTS

Correlation between cation effects on fluorescence and detergent fractionation

The fluorescence yield of isolated chloroplasts is strongly influenced by cation concentrations of the solutions in which the plastids are suspended [2, 3]. Addition of sequentially increasing amounts of Mg^{2+} or K^+ to pea chloroplasts suspended in 10 mM NaCl, 1 mM Na Tricine, and 0.02 mM DCMU caused corresponding increases in steady state fluorescence (F_m). The values of F_m measured after various cation additions are plotted in Figs. 1 and 2. Maximal cation stimulation occurred after addition of a final concentration of 3 mM Mg^{2+} or 150 mM K^+. Addition of 3 mM Mg^{2+} to a reaction mixture containing 150 mM K^+ did not induce any

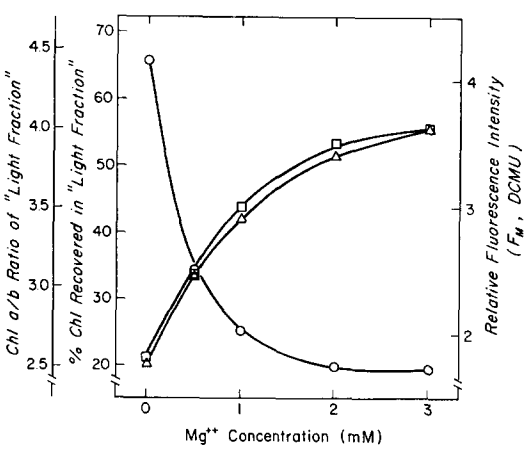

Fig. 1. The effect of various concentrations of $MgCl_2$ on fluorescence yield and upon chlorophyll recovery in a light fraction following detergent fractionation of isolated chloroplasts. Measurements of fluorescence (□—□) were in reaction mixtures of 10 mM NaCl, 1 mM Na-Tricine (pH 7.8), $1 \cdot 10^{-5}$ M DCMU, and 10 μg chlorophyll per ml. Excitation light was at 440 ± 5 nm; emission was monitored at 685 ± 5 nm. The sample was stirred continuously. Small aliquots of a 1 M stock solution of $MgCl_2$ were added to give the final salt concentrations indicated. Detergent fractionation (described in text) involved digitonin incubation with chloroplasts in 10 mM NaCl, 1 mM Na-Tricine and the indicated level of $MgCl_2$, followed by a single centrifugation of the mixture at 40 000 $\times g$. The material recovered in the 40 000 $\times g$ supernatant (the light fraction) was analyzed for total chlorophyll content (○—○) and for chlorophyll *a*/*b* ratio (△—△). The chlorophyll concentration in the initial digitonin-chloroplasts incubation solution prior to centrifugation was 600 μg/ml with a chlorophyll *a*/*b* ratio of 2.5.

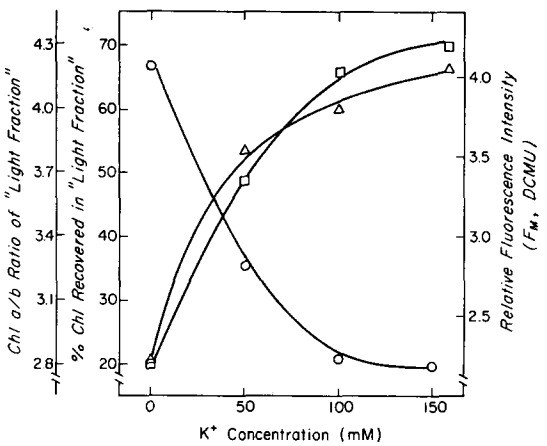

Fig. 2. The effect of various concentrations of KCl on fluorescence yield and upon chlorophyll recovery in a light fraction following detergent fractionation. Measurements of fluorescence (□—□) were exactly as described in Fig. 1, with the exception that aliquots of a 4 M KCl solution were added to give the indicated K^+ concentrations. Detergent treatments were as described in Fig. 1, except that various levels of KCl replaced $MgCl_2$ during detergent treatment. ○—○, total chlorophyll content; △—△, chlorophyll *a*/*b* ratio.

further fluorescence yield increase. These data are in direct agreement with earlier studies of Murata [16] in which he concluded that cations suppress exciton transfer from Photosystem II to Photosystem I.

Figs. 1 and 2 contain data from another series of experiments in which certain aspects of chloroplast fractionation studies were carried out under conditions nearly identical to those used for fluorescence measurement. In these experiments, chloroplasts suspended in 10 mM NaCl and 1 mM Na Tricine were incubated with various concentrations of monovalent or divalent cations, as indicated, for 15–30 min at room temperature. (This incubation period was found to be necessary for obtaining reproducible results in the following analysis. It might be noted that this is only slightly longer than time needed for ions to cause maximum stimulation of fluorescence of chloroplasts previously in low-salt media.) Digitonin was then added to each sample and, after further incubation, the samples were centrifuged at $40\,000 \times g$ to yield a heavy pellet and a light supernatant fraction. It was found that cation concentrations had a dramatic effect on both the amount of chlorophyll recovered in the light fraction and the chlorophyll *a*/*b* ratio of this fraction (Figs. 1 and 2). In the absence of Mg^{2+} or K^+, over 60 % of the sample chlorophyll was recovered in the light fraction; the chlorophyll *a*/*b* ratio of this material was approximately the same as the starting chloroplast sample. In samples in which the cation concentrations were increased during detergent treatment, the amount of chlorophyll in the light fraction decreased while the chlorophyll *a*/*b* ratio of the light material increased. This phenomena saturated at about 3 mM Mg^{2+} or 150 mM K^+. Treatments containing 3 mM Mg^{2+} plus 150 mM K^+ did not yield results that differed significantly from those with saturating amounts of either of the individual cations.

Photochemical activities were determined for samples obtained following detergent fractionation under conditions described for Figs. 1 and 2. In all cases, the

TABLE I

CHARACTERIZATION OF HEAVY AND LIGHT FRACTIONS RECOVERED AFTER DETERGENT TREATMENT IN HIGH OR LOW SALT CONDITIONS

Samples were treated with detergents as described in Materials and Methods. Centrifugation at 40 000 × *g* yielded a pellet (heavy fraction) and supernatant (light fraction). The 40 000 × *g* supernatant solution was pelleted at 144 000 × *g* to concentrate the sample prior to assay.

Mg^{2+} and K^+ concentration during detergent fractionation	Sample recovered by differential centrifugation	Chlorophyll *a*/*b* ratio	Recovery (%)	Photosystem II activity*	Photosystem I activity**
–	Control chloroplasts	2.6	–	304	390
3 mM Mg^{2+}, 100 mM K^+	Heavy fraction	1.9	78	352	302
	Light fraction	4.7	22	15	923
0 Mg^{2+}, K^+	Heavy fraction	2.5	38	280	377
	Light fraction	2.5	62	263	371

* μmol DCPIP reduced /mg chlorophyll per h (Diphenyl carbazide → DCPIP electron transport).
** μmol O_2 consumed/mg chlorophyll per h (Plastocyanin → methyl viologen electron transport).

chlorophyll *a*/*b* ratio of the light fraction was inversely related to the amount of Photosystem II activity present. When the *a*/*b* ratio was low, there were high rates of Photosystem II in the light submembrane fraction and vice versa. Membrane fractionation in saturating amounts of cations resulted in a heavy fraction enriched in Photosystem II and a light fraction enriched in Photosystem I but nearly devoid of Photosystem II (Table I). This is similar to the pattern of photosystem separation which has been reported in many earlier studies [7, 17]. In contrast, the distribution of activities of Photosystems I and II were equal in light and heavy fractions following fractionation in the absence of Mg^{2+} or K^+ (Table I).

Structural characterization of submembrane fragments

We have previously demonstrated that the particle size of detergent-derived submembrane fractions can be approximated by ultrafiltration studies [18]. Table II shows the filtration analysis of heavy and light fractions from chloroplasts fragmented in the presence and absence of Mg^{2+} and K^+. The Photosystem II-enriched high-salt heavy fraction did not penetrate a large pore (1000 Å) filter. This is consistent with our earlier observation that digitonin-derived Photosystem II preparations obtained in the presence of salts maintain a membranous structure [17]. The light Photosystem I-containing material from high-salt chloroplasts was retained only on small (100 and 65 Å pore diameter) filters. This is also in agreement with earlier studies which showed Photosystem I fractions to be comprised of small subunits which can be caused to aggregate to form membranous vesicles under appropriate conditions [17, 18]. The heavy fraction after low-salt treatment did not penetrate even the large pore filter. Since this sample had the same photochemical activity and chlorophyll *a*/*b* ratio as the control chloroplasts, we interpret the ultrafiltration data to mean that the preparation contained relatively large membrane fragments which were not completely digested by detergent action. The low-salt light fraction, even though it contained both Photosystem I and II activities and was nearly identical to the control chloroplasts in chlorophyll *a* and *b* content, was not retained by a 1000 Å pore size filter but was largely retained by the 1000 Å pore diameter filter. We interpret these

TABLE II

RETENTION OF VARIOUS MEMBRANE FRAGMENTS ON ULTRAFILTERS

Chloroplasts were treated as described in Table I. The pellets were resuspended in the respective resuspension solutions used for detergent treatment. All samples were subjected to filtration in an Amicon ultrafiltration cell operated at 10 lb/inch^2 N_2 gas pressure with constant stirring at 20 °C. A comparison of chlorophyll concentrations in the initial solution and in the filtrate allowed a determination of the percent sample retained by each of the filters. Filters used were Sartorius 11309, Sartorius 11311 and Amicon SM100A with pore sizes of 0.1, 0.01, 0.0065 μm respectively.

Mg^{2+} and K^+ concentration during detergent fractionation	Sample recovered by differential centrifugation	Chlorophyll retention (%)		
		Pore diameter of filter (Å):		
		1000	100	65
3 mM Mg^{2+}, 100 mM K^+	Heavy fraction	99–100	–	–
	Light fraction	15	74	99
0 Mg^{2+}, K^+	Heavy fraction	99–100	–	–
	Light fraction	25	80	99

latter data to indicate that the light fraction formed in the absence of Mg^{2+} or K^+ is derived from thoroughly disrupted or fragmented regions of the chloroplast lamellae, and consists of dispersed photochemical complexes. It should be noted that the exclusion limit for macromolecules by the ultrafilters is not extremely sharp, especially when the macromolecules are not rigid spheres. The pore size of the filter giving retention of a particular sample therefore gives only an approximation of the actual particle size of that sample.

Separation of pigmented complexes by sucrose gradient centrifugation

Since the low-salt light fraction contained dispersed submembrane fragments which had both Photosystem I and II activity, it was of interest to subject this

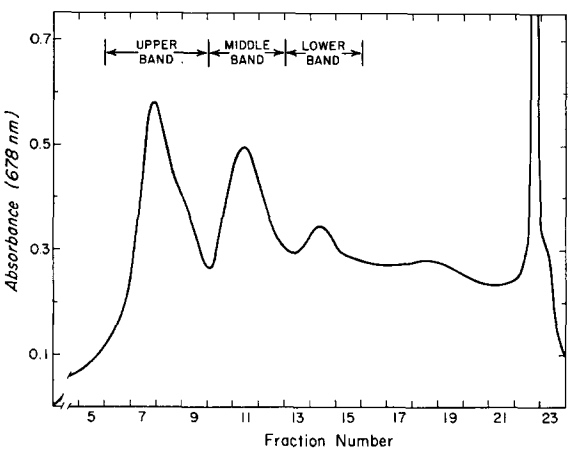

Fig. 3. Elution profile of a sucrose gradient tube scanned at 678 nm. The solution in the gradient tube was forced out of the top of the tube through a specially constructed tube cap, passed through a flow-monitor cuvette cell in a Coleman 124 spectrophotometer, and then collected in equal volume fractions.

TABLE III

DISTRIBUTION OF PHOTOCHEMICAL ACTIVITIES WITHIN THE THREE BANDS RECOVERED FROM SUCROSE GRADIENT SEPARATION OF THE 40 000 × *g* SUPERNATANT MATERIAL FOLLOWING LOW-SALT DETERGENT TREATMENT OF CHLOROPLAST LAMELLAE.

Sucrose gradient bands are designated as defined in Fig. 3. The detergent-chloroplast incubation medium contained 10 mM NaCl, 1 mM Na-Tricine (pH 7.8).

Sample	Photosystem II activity (μmol DCPIP reduced/ mg chlorophyll per h)	Photosystem I activity (μmol O_2 consumed/ mg chlorophyll per h)
Control Chloroplast	240	348
40 000 × *g* supernatant	200	330
Sucrose gradient fractions:		
Upper Band	14	30
Middle Band	120	822
Lower Band	396	726

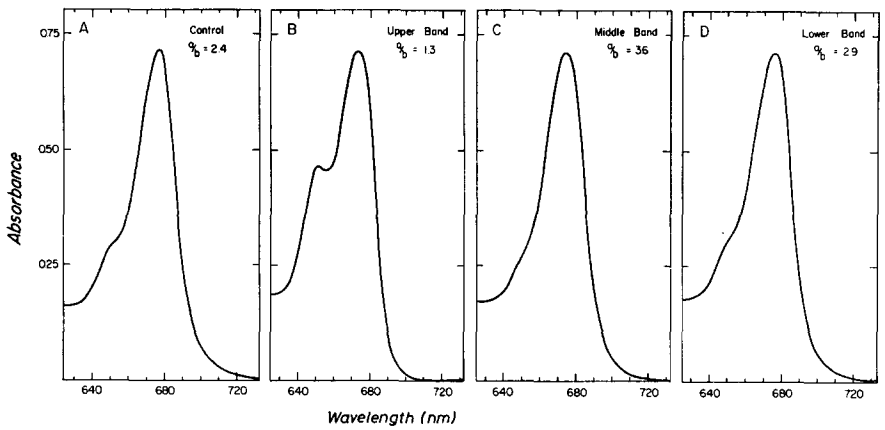

Fig. 4. Absorption spectra for control chloroplasts and for chlorophyll-containing samples collected from sucrose gradient tubes. The samples are labeled as shown in Fig. 3. Chlorophyll *a*/*b* ratios calculated from acetone extracts are indicated.

material to sucrose gradient centrifugation in an attempt to separate various components. An elution profile of one such gradient tube scanned for chlorophyll (absorbance at 678 nm) is shown in Fig. 3. A portion of the sample loaded on the gradient apparently aggregated and pelleted to the bottom of the tube, leaving residual chlorophyll-containing material throughout the bottom third of the gradient (background absorbance in fractions 16–23). This material is not considered in the following discussion. However, three distinct chlorophyll containing bands were visible near the top of the tube. Fractions 6–9, 10–12, and 13–15 were saved from each gradient tube. All samples in each grouping were pooled and identified as upper, middle, or lower bands, respectively, in the following experiments.

Chlorophyll *a*/*b* ratios and photochemical activities of the three bands collected from sucrose gradient tubes are shown in Table III and Fig. 4. The top band had a very low chlorophyll *a*/*b* ratio and had almost no Photosystem I or II activity. (It should be noted that peaks of the elution profile of Fig. 3 are overlapping. It is likely that the limited photochemical activity of the top band is due to overlapping contamination of material in the middle band.) The middle band had a reduced chlorophyll *b* content and was heavily enriched in Photosystem I activity. The lower band contained most Photosystem II activity, but also showed high rates of Photosystem I activity, and was partially chlorophyll *b*-depleted.

A comparison of a portion of the chlorophyll absorption spectra for control chloroplasts and the three bands from the gradient are shown in Fig. 4. The abundance of chlorophyll *b* in the upper band is clearly shown by the prominent peak at 652 nm. The red wavelength maximum for chlorophyll *a* was at 673 nm. It should be noted that there was little absorbance at long wavelengths (> 690 nm) in the upper band sample. In other studies, it was found that addition of up to 10 mM Mg^{2+} to this sample did not change absorption characteristics. The absorption spectra of the middle and lower band are similar to those of control chloroplasts with the exception that they are partially chlorophyll *b*-depleted.

Uncorrected low-temperature fluorescence emission spectra for the upper

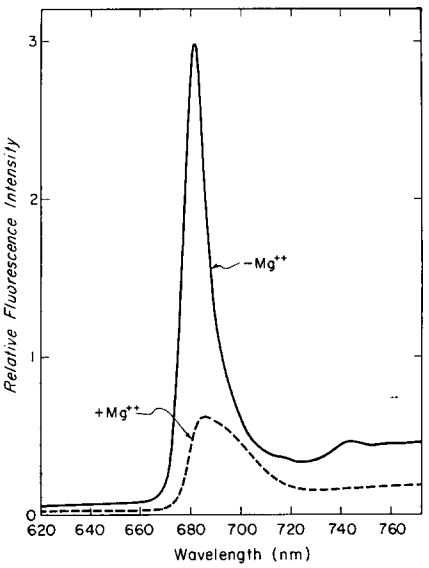

Fig. 5. Fluorescence emission spectra measured at liquid nitrogen temperature for the chlorophyll-containing material collected in the upper band of the sucrose gradient (see Fig. 3). Fluorescence excitation was at 440 nm $\pm$5. The upper trace was a sample (10 μg chlorophyll) in 10 mM NaCl and 1 mM Na Tricine (pH 7.8). The lower trace was an identical sample to which a small aliquot of 1 M $MgCl_2$ had been added (to give 10 mM final concentration) 10 min before freezing.

band sample are shown in Fig. 5. In the absence of divalent cations, a major emission peak at 682 nm was observed; there was little fluorescence at long (> 690 nm) wavelengths. Addition of 10 mM Mg^{2+} to the sample several minutes prior to freezing for fluorescence analysis resulted in a severe quenching of fluorescence (Fig. 5). If the sample was pretreated with 1 % glutaraldehyde prior to Mg^{2+} addition, the cations had virtually no effect on fluorescence yield. This may indicate that Mg^{2+} quenching involves a conformational change of the pigment · protein complex which is blocked by glutaraldehyde fixation. The results are equivocal, however, since glutaraldehyde treatment itself introduces quenching effects.

Electrophoretic analysis of sucrose gradient fractions

We have subjected control chloroplasts and each of the sucrose gradient samples described above to SDS solubilization and polyacrylamide gel electrophoresis (Fig. 6). As has been previously described, control chloroplast membranes can be resolved into three pigmented zones which have been identified as the *P*-700-chlorophyll *a* protein, a light-harvesting chlorophyll *a*/*b* · protein and a free pigment zone [19, 20].

When the upper band of the sucrose gradient was examined by the gel technique (Fig. 6), the *P*-700-chlorophyll *a* protein band was found to be entirely missing, whereas the light-harvesting chlorophyll *a*/*b* · protein band was greatly enriched. In the middle and lower sucrose gradient samples, the light-harvesting pigment · protein portion of the gel was greatly diminished in chlorophyll content whereas the *P*-700-chlorophyll *a* protein and free-pigment content were increased.

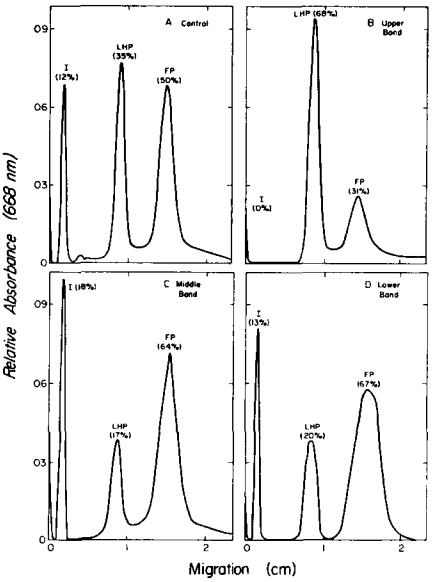

Fig. 6. Spectrophotometric scans (at 668 nm) of polyacrylamide gels containing SDS-solubilized chlorophyll · protein complexes. (A), control chloroplasts; (B–D), samples derived from sucrose gradient fractionations (see Fig. 3). (I, *P*-700 chlorophyll *a* protein; LHP, light-harvesting chlorophyll *a*/*b* protein; FP, free pigment.) Numbers in parenthesis refer to calculated areas under each of the peaks; the values represent the percentage of total chlorophyll obtained in the various components.

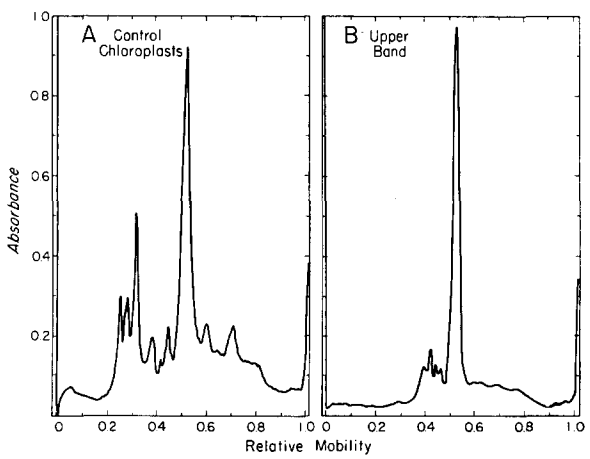

Fig. 7. Spectrophotometric scans (at 580 nm) of chloroplast membrane proteins solubilized in SDS, separated on polyacrylamide gels, and then stained with Coomassie blue. A, control chloroplast lamellae which had been washed with EDTA. B, the top band from the sucrose gradient (see Fig. 3).

We have also analyzed the protein content of lipid-extracted material from each of the sucrose gradient bands by disc gel electrophoresis. In these experiments, the chloroplasts were washed twice with 1 mM EDTA prior to detergent treatment to remove membrane bound coupling factor and ribulose diphosphate carboxylase [21]. The polypeptide profiles observed for control chloroplasts (Fig. 7A) are similar to those previously described [13, 22]. As was noted in these earlier studies, a polypeptide of approximately 24 000 daltons (relative mobility (R_m), 0.52 in our data) is the major protein constituent of the solubilized membrane preparation. The middle and lower bands of the sucrose gradient gave polypeptide profiles (not shown) which were very similar to those of the control chloroplasts, with the exception that the 24 000 dalton polypeptide species (R_m, 0.52) was reduced in content. In contrast, the upper band from the sucrose gradient tubes had a very limited polypeptide complement; the data in Fig. 7B show that it consisted almost entirely of a single polypeptide with a relative mobility of 0.525 (corresponding approximately to 24 000 daltons), in addition to four minor protein bands of slightly higher molecular weight. (It should be noted that the largest of these minor polypeptides, $R_m = 0.39$, may be a dimer of the 24 000 M_R component, since the relative area under this peak could be greatly increased by lowering the SDS concentration during solubilization treatment with a corresponding, but opposite change in the $R_m = 0.525$ peak.)

Detergent fractionation of chloroplasts deficient in the light-harvesting chlorophyll a/b protein

It has previously been demonstrated that exposure of dark-grown pea plants to intermittent illumination (cycles of 2 min light, 2 h dark), results in development of chloroplasts which contain chlorophyll *a* but no chlorophyll *b* [4]. These plastids are also deficient in the light-harvesting chlorophyll a/b protein [5]. We have examined the pattern of detergent action in fractionation of the plastids from the plants grown in intermittent light. As is shown in Table IV, inclusion of $MgCl_2$ during fractionation had no significant effect on the distribution of chlorophyll during differential centrifugation; in both the presence or absence of Mg^{2+}, the major portion of the chlorophyll derived from intermittent light chloroplasts was recovered in the light fraction. Other studies (data not presented) showed that Photosystem I and II were found in both heavy and light fractions with the specific activities of both being approximately

TABLE IV

A COMPARISON OF THE PATTERN OF DETERGENT FRACTIONATION OF INTERMITTENT LIGHT PLASTIDS, WHICH LACK THE LIGHT-HARVESTING CHLOROPHYLL *a/b* PROTEIN [4, 5], AND NORMAL CHLOROPLASTS.

Conditions for fractionation were as described in Materials and Methods. The supernatant after centrifugation at 40 000 × *g* is indicated as a light fraction.

Salt concentration during detergent incubation	Chlorophyll recovery in light fraction (%)	
	Intermittent light plastids	Control chloroplasts
10 mM NaCl, 1 mM Na-Tricine	75	73
10 mM NaCl, 1 mM Na-Tricine, 10 mM $MgCl_2$	68	14

TABLE V

DISTRIBUTION OF CHLOROPHYLL ON A SUCROSE GRADIENT FOLLOWING DETERGENT FRACTIONATION OF INTERMITTENT LIGHT PLASTIDS AND CONTROL CHLOROPLASTS

Sample preparation prior to gradient analysis was as described in Table IV using 0 $MgCl_2$ during fractionation. Sucrose gradients were prepared as in Materials and Methods. Fractions were collected as described in Fig. 3. Fractions 6–9 were designated as the upper band, fractions 10–12 as middle band and fractions 13–15 as lower band for both tubes, even though the intermittent light plastid sample did not contain an upper band by visual examination and the middle and lower bands visually merged into one broad band. The amount of chlorophyll recovered in each of the three bands was determined and is expressed as a percentage of the total amount recovered in all three bands of that gradient tube.

Gradient fraction	Chlorophyll recovered in band	
	Total chlorophyll recovered in 3 bands (% of total)	
	Intermittent light plastids	Control chloroplasts
Upper band	4	28
Middle band	64	39
Lower band	32	33

equal to the unfractionated chloroplasts. Control chloroplasts (Table IV), however, were markedly affected by Mg^{2+} during fractionation (in the same pattern as was shown in Fig. 1 and Table I above).

The pigmented material recovered from intermittent light plastids and control plastids in the light fraction after detergent treatment in the absence of Mg^{2+} (described in Table IV) was further utilized for sucrose gradient analysis. The results of these studies are shown in Table V. The sample derived from intermittent light-grown chloroplasts completely lacked the upper chlorophyll band on the sucrose gradient when examined visually; the 4 % chlorophyll reported in Table V to be recovered in this band is due to overlap of the upper and middle sucrose gradient bands. Most of the chlorophyll in the intermittent light plastids sample was recovered in a wide middle band. This band, when examined for photochemical activities, was found to contain both Photosystems I and II.

DISCUSSION

We have demonstrated that the presence of cations during detergent-chloroplast incubation has profound effects on the pattern of release of submembrane fragments. The amount of either monovalent or divalent cations which were needed to elicit these effects was directly correlated to the cation concentrations required to regulate excitation energy distribution between Photosystems II and I. We believe that both these phenomena reflect submembrane conformation changes in which cations alter the interaction of a light harvesting pigment · protein complex with the Photosystem II reaction center complex. In the following discussion, we will first relate our fractionation analysis to previous research on detergent-derived submembrane fragments. We will then correlate the fractionation data to the effects of cations on exciton transfer from Photosystem II to Photosystem I.

Solubilization of a photochemically inactive pigment-protein from chloroplast lamellae

When chloroplast lamellae are detergent treated in the absence of divalent cations or at very low monovalent cation levels, a major portion of the membranes is dissociated into small subunits (about 100 Å; Table II) which stay in a light supernatant fraction following differential centrifugation. We have shown that a photochemically inactive complex with a chlorophyll *a*/*b* ratio of 1.3 can be purified from this solubilized material by sucrose gradient separation. This chlorophyll *b*-enriched complex was not present in greening plastids grown under intermittent light (Table V), even though the intermittent light plastids had full photochemical activity [4]. We therefore conclude that the chlorophyll *b*-enriched complex is not required for photochemical activity. We hypothesize that it serves as a light-harvesting complex in the mature chloroplast lamellae.

Relationship of the light-harvesting complex to previously studied chlorophyll proteins

Thornber and associates (see ref. 20) have extensively analyzed a light-harvesting chlorophyll *a*/*b* protein extracted from chloroplasts with anionic detergents and, in some instances, purified on polyacrylamide gels. When our light-harvesting complex (top band of sucrose gradient, Fig. 6) was subjected to similar polyacrylamide gel analysis, nearly 70 % of the chlorophyll in the gel was associated with the light-harvesting chlorophyll *a*/*b* protein band (the remaining portion being in a free pigment band). The red absorption maxima of the chlorophyll in our light-harvesting complex preparation (652 and 673 nm) were very similar to the reported values for the chlorophyll *a*/*b* protein (653 and 672 nm). Both the light-harvesting complex and the light-harvesting chlorophyll *a*/*b* protein of Thornber and coworkers exhibit a single fluorescence emission peak with maxima at 682 nm for the light-harvesting complex (Fig. 5) and 685 nm for the chlorophyll *a*/*b* protein [20]. The protein moiety of the light-harvesting chlorophyll *a*/*b* protein is thought to be a polypeptide of about 25 000 daltons [20]. Our light-harvesting complex preparation is greatly enriched in a polypeptide of this size range (Fig. 7B), but in addition contains three or four other polypeptides in minor amounts. (The minor polypeptide bands may be contaminating proteins banding at the same level as the chlorophyll · protein on the gradient.)

Based on the similarities in characteristics of the light-harvesting chlorophyll *a*/*b* protein extracted by anionic detergents, and our light-harvesting complex released by digitonin treatment of low-salt lamellae, it seems certain that the preparations are in most respects identical. Since our light-harvesting complex is a submembrane fragment of relatively large size (approx. 100 Å, based on ultrafiltration results), it is likely to be an aggregate of the light-harvesting chlorophyll *a*/*b* proteins described by Thornber and associates [20]. We conclude that the aggregate represents a structural complex which exists within the membrane, since its release is attained by mild detergent treatments.

Isolation of the light-harvesting complex in relation to previous fractionation studies

Most previous non-ionic detergent (digitonin or Triton X-100) fractionation experiments have shown that a heavy Photosystem II fraction, which is enriched in chlorophyll *b*, can be separated from a light Photosystem I fraction [6–8, 17]. We have found that this separation pattern is highly dependent on cation concentrations during detergent treatment (Table I, Figs. 1 and 2) and have asked why a light

Photosystem II (recovered in the 40 000 $\times g$ supernatant; Table I) is converted in the presence of cations into a heavy preparation which pellets at 40 000 $\times g$. The answer which we suggest is that interactions between Photosystem II complexes and the light-harvesting complexes are regulated by cations. In the presence of approx. 3 mM Mg^{2+} or higher amounts (approx. 150 mM) K^+, there is strong interaction between the Photosystem II and the light-harvesting complexes. It seems likely that there could be multiple sites of interaction between each of the complexes, thus resulting in extensive cross-linking between subunits and formation of lamellar arrays. These sheet-like submembrane fragments would behave as a heavy fraction in differential centrifugation separation. Lamellar aggregates in the chlorophyll *b*-enriched Photosystem II fraction were, in fact, observed in earlier electron microscopy studies [17]. In the presence of low cation concentrations, however, we believe the interactions between Photosystem II reaction center and light-harvesting complexes must be primarily hydrophobic; these interactions are readily disrupted by detergents, giving rise to separate small complexes that behave as a light fraction during centrifugation.

If interactions between the light-harvesting complex and Photosystem II complexes are necessary in order to recover a heavy fraction during detergent fractionation, as outlined above, then it should be expected that plastids deficient in the light-harvesting complex would not yield normal Photosystem I-Photosystem II separations by differential centrifugation. This has indeed been found to be true; intermittent-light plastids which are deficient in the light-harvesting complex (Table V) do not yield a Photosystem II enriched heavy fraction even in the presence of high cation concentrations (Table IV). It seems likely that previously reported failures to achieve normal separation of Photosystems I and II following detergent fractionation of a chlorophyll *b*-deficient algal mutant chloroplast [10] and a mutant of barley lacking in chlorophyll *b* [23] were also due to the absence of the light-harvesting complex in these plastids.

Involvement of the light-harvesting complex in cation-regulation of excitation energy distribution

Cations are known to regulate the distribution of absorbed excitation energy between Photosystems I and II. More specifically, cations are thought to block exciton transfer from Photosystem II to Photosystem I, thus stimulating fluorescence yield at room temperature [2, 3, 16]. We have shown that when increasing concentrations of cations are present during detergent fractionation, there are increasing amounts of a Photosystem II and chlorophyll *b*-enriched submembrane material recovered in the heavy fraction following differential centrifugation. When nearly identical experimental conditions were used for fluorescence measurements (in the absence of detergents), the cation requirement for stimulating fluorescence yield was nearly identical to the cation requirement needed to generate a heavy Photosystem II preparation (Figs. 1 and 2). Both phenomena appear to saturate at the same Mg^{2+} or K^+ concentrations and neither is significantly stimulated above this maximal level by the simultaneous addition of K^+ and Mg^{2+}. We conclude that the same cation-induced interactions between Photosystem II and the light-harvesting complex which are involved in creating a heavy membranous fraction are also intimately involved in regulating excitation-energy distribution between the photochemical complexes within the membrane.

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