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IMPROVEMENT IN SEPARATION OF SYSTEM I AND SYSTEM II PARTICLES OF PHOTOSYNTHESIS OBTAINED BY DIGITONIN TREATMENT

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SUMMARY

By a combined use of digitonin treatment and subsequent centrifugation on a linear sucrose density gradient, the whole green material of the chloroplast lamellae was separated into System I and System II particle fractions, leaving no other fractions of intermediate properties at the final step of separation.

Each of these particle fractions obtained had properties characteristic of System I or System II with respect to the molar ratio of chlorophyll *a*/chlorophyll *b*, the content of P700, the fluorescence emission spectrum at −196°, photoreduction activities with ferricyanide and $NADP^+$, and induction of fluorescence.

About 40 and 50% of the total chlorophyll in the original chloroplasts were recovered in System I and System II particles, respectively. Only small amounts of total chlorophyll (less than 10%) were found as free chlorophyll detached from the lamellae through the digitonin treatment.

These results support the view that the lamellae of chloroplasts are composed of about equal amounts of System I and System II particles on a chlorophyll basis.

INTRODUCTION

Since the work of BOARDMAN AND ANDERSON[1] in 1964, attempts have been made by many investigators to separate two particle units, such that a single unit would correspond to one of the two photochemical systems of photosynthesis[2–5]. Isolation of two particle fractions from chloroplasts, each retaining some of the original photochemical activities as well as the chemical compositions characteristic of Systems I and II, has been reported. These results supported the view that the two photochemical systems of photosynthesis exist as such particulate entities in the lamellae of chloroplasts. However, the yields of the System I and System II particles in the fractionation procedures thus far reported were rather low, a greater part of the green material being discarded in the course of isolation. Also, other fractions of intermediate nature with respect to both the chemical compositions and photochemical activities were found. There was no experimental evidence as to whether such a particle frac-

Abbreviation: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea.

tion represented a mixture of the isolated System I and System II particles or a fraction consisting of particles in which the two systems remained incompletely separated.

The aim of the present study was to obtain a high resolution of the whole lamellar material of the chloroplasts into two particle units, each retaining its photochemical activities. The experimental results are described, and the conditions required for efficient separation and high yields of the two particle fractions are also discussed.

MATERIALS AND METHODS

Chloroplasts were isolated from spinach leaves using a medium containing phosphate buffer (pH 7.8), 0.4 M sucrose and 0.01 M NaCl. The chloroplasts were suspended in 0.05 M Tricine buffer (pH 7.3) containing 0.15 M KCl (chlorophyll concentration, 0.25–0.3 mg chlorophyll/ml) and incubated at 0° for 30 min with the addition of digitonin (final concentration, 0.5%) which had been purified by crystallization from hot ethanol solution. The mixture (1.5 ml) was then fractionated by centrifugation at 51000 × *g* for 50 min on a linear sucrose density gradient (sucrose 15–55%, 0.05 M Tricine buffer (pH 7.3) containing 1% Nikkol*).

Chlorophyll was determined by the method described by ARNON[6]. The content of chlorophyll in each fraction of sucrose density gradient centrifugation was expressed as the absorbance at 678 nm corresponding to the red absorption maximum of the particle fractions. The absorbance was measured in a cuvette 1 cm × 4 cm × 0.4 cm (light path, 0.4 cm) in a Shimadzu multipurpose spectrophotometer. It was confirmed in an additional experiment that, within the limits of experimental errors, the absorbance at 678 nm thus measured was approximately proportional to the amount of total chlorophyll in each fraction. Under the conditions of the present experiment, one unit of absorbance at 678 nm of the particle fractions (light path, 0.4 cm) corresponded to the chlorophyll concentration of 42 μg chlorophyll/ml. The amount of P700 was estimated by measuring the absorbance change at 700 nm before and after the additions of ascorbate and ferricyanide. The measurements were performed in an Aminco-Chance dual wavelength spectrophotometer equipped with a photomultiplier RCA 7102. The difference spectrum obtained by this method was in good agreement with the oxidation–reduction difference spectrum of P700 in acetone-treated chloroplasts originally reported by KOK[7]. The relative amount of P700 was given by the ratio, $\Delta A_{700\text{ nm}}/A_{678\text{ nm}}$.

Photoreductions of ferricyanide and $NADP^+$ were measured by following the changes in absorbance at 420 and 340 nm, respectively. Photoreduction of $NADP^+$ was carried out anaerobically in the presence of glucose and glucose oxidase[8] (*cf.* legend for Fig. 3). Ferredoxin, ferredoxin–$NADP^+$ reductase and plastocyanin were prepared from spinach by the standard methods[9–11]. Light furnished from a 600-W iodine lamp filtered through a water filter, a cut-off filter (Toshiba VR-60), and an infrared-absorbing filter (Hoya HA 50) was used as the actinic light (> 600 nm).

* Nikkol: polyoxyethylene hydrogenated castor oil derivatives; kindly afforded by Nikko Chemicals Co. The experimental results shown in this report were obtained in the presence of this material. However, similar results were obtained irrespective of the presence or absence of this detergent.

The incident light intensity was $5 \cdot 10^5$ ergs$\cdot$cm$^{-2}\cdot$sec^{-1}. Interference filters having transmission maximum at 420 and 340 nm were placed in front of the photomultiplier.

The fluorescence emission spectra at $-196°$ were measured by the method described by MURATA *et al.*[12]. Excitation light was furnished by a 150-W tungsten lamp, filtered through an infrared-absorbing filter IRO-1A (Toshiba) and two blue filters, VB 46 (Toshiba) and B460 (Hoya). The transmission maximum of the filter combination lay at 470 nm (32% transmission). The fluorescence was analyzed using a Bausch and Lomb monochromator equipped with Toshiba VR-63 cut-off filter (half band width, 2.5 nm). Fluorescence emission spectra were not corrected for the spectral sensitivity of the equipment used (for corrections, see MURATA *et al.*[12]; the same equipment was used in the present study). The concentration of chlorophyll in each measurement was made below 10 nmoles chlorophyll/ml.

The time-courses of fluorescence emission were measured by the methods reported by MURATA[13]. The excitation light was furnished by a Baush and Lomb grating monochromator (475 nm, 20-nm half band width) equipped with a Hoya B 460 filter. The incident light intensity was 250 ergs$\cdot$cm$^{-2}\cdot$sec^{-1}. The fluorescence was detected at an angle of 90° to the excitation light beam, using a photomultiplier R236 (Hamamatsu TV) equipped with an interference filter at 685 nm to exclude the excitation light. In the measurements of fluorescence induction, the concentrations of chlorophyll in the particle samples were made below 2 nmoles chlorophyll/ml.

RESULTS

On centrifugation of the digitonin-treated chloroplasts on a sucrose density gradient at 51000 $\times$ *g* for 50 min, the whole green material was separated into three distinct bands, which will be designated from the top downwards, as F-1, F-2 and F-3 (see Fig. 1). On further centrifugation for another 50-min period, the F-2 band was found to move down to the position of F-3, while the F-1 band remained at almost the same position. The chemical compositions and the photochemical activities of these fractions were investigated with the purpose of the assignment of the photochemical Systems I and II.

Chemical compositions of the particle fractions

The distributions of chlorophyll and P700 in successive fractions of density gradient centrifugation are shown in Fig. 1. In the upper part of the figure, the relative values for P700/chlorophyll are also given. 86% of the P700 present in the digitonin-treated mixture was recovered in F-1. The contents of P700 in F-2 and in F-3 were 10 times lower than that in F-1. In fractions on the left-hand side of the peak of F-1 (Fractions 19–23) in the figure, the ratio for P700/chlorophyll was almost constant, amounting to 2.5 times as high as that in the original chloroplasts (see upper curve). In top fractions of F-1 (Fractions 24–26) lying further to the right in the figure, the ratios sharply decreased. Considering also the findings of fluorescence experiments to be described in a later section, this observation suggested a contamination of free chlorophyll detached from the green particles through digitonin treatment. The amounts of the free chlorophyll contained in these fractions (24–26) were calculated on the assumption that the ratio for P700/chlorophyll is constant throughout the F-1 fraction particles (see the curve in dotted line in Fig. 1). The contents

of chlorophyll in F-1, F-2 and F-3 were, respectively, 40% (*plus* 7% free chlorophyll), 43% and 9.7%, of the chlorophyll present in the digitonin-treated mixture. Almost complete recoveries (about 99%) of both chlorophyll and P700 during these procedures should be mentioned. The molar ratios of chlorophyll *a*/chlorophyll *b* were 5.2 in F-1, 2.0 in F-2 and 2.2 in F-3 (3.0 in the digitonin-treated mixture).

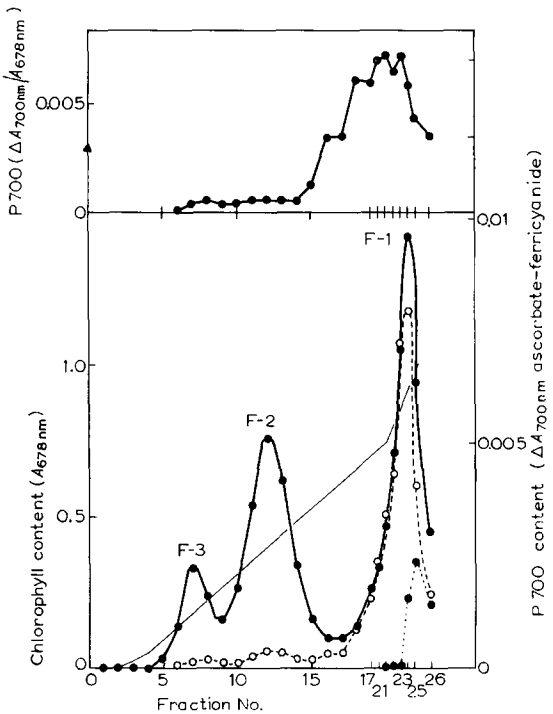

Fig. 1. Distributions of chlorophyll and P700 in various fractions of sucrose density gradient centrifugation. The volume of Fractions 19–25 was one-half that of the other fractions. The chlorophyll contents are shown as the absorbance at 678 nm (red absorption peak of the particles). ●—●, absorbance at 678 nm; ○—○, ΔA at 700 nm (ascorbate–ferricyanide); ●----●, free chlorophyll (calculated, see text). In the upper part of the figure are shown the values for P700/chlorophyll ($\Delta A_{700\ nm}/A_{678\ nm}$). ▲ P700/chlorophyll for the digitonin-treated mixture and original chloroplasts. Thin line (———) represents the concentration of sucrose (%) in the density gradient.

In order to determine the form of chlorophyll *a* contained in these fractions, the fluorescence emission spectra were measured at −196°. In spinach chloroplasts, there are three emission bands, F684, F695 and F735. The two former are ascribed to System II and the last one to System I (refs. 12, 15). The positions of these emission bands were not altered by the digitonin treatment, although there were marked changes in the relative heights of the peaks in the emission spectra (Fig. 2). The emission spectrum of F-1 was characterized by the prominence of F735 (Fig. 2e), in contrast to the findings with F-2 and F-3 in which F684 and F695 dominated (Figs. 2c and 2d). There was no significant difference in this respect between F-2 and F-3. The top fractions (24–26) of F-1, having a low P700/chlorophyll value (see above), produced an emission spectrum different from that for the rest of the fractions (Fig. 2f). There was a shorter wavelength band having a maximum at 680 nm besides

the one at 735 nm; the emission band at 680 nm is due to the free chlorophyll present in these fractions[15,16]. It should be mentioned in this connection that the occurrence of the 680-nm band in the top fractions of F-1 coincides with that of the lower ratio of P700/chlorophyll. The relative heights of the three emission bands in the various fractions are summarized in Table I.

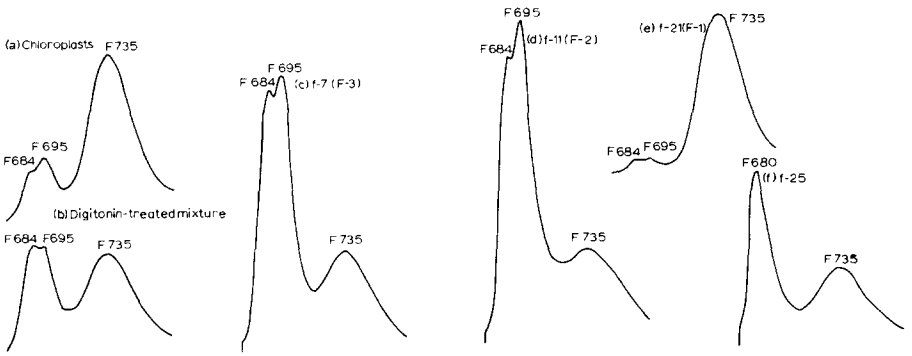

Fig. 2. Fluorescence emission spectra at −196° in various fractions. Concentrations of chlorophyll in the particle fractions in the measurements were less than 10 nmoles chlorophyll/ml. Fraction numbers correspond to those given in Fig. 1. Strict comparisons between these spectra cannot be made with respect to the absolute intensities of fluorescence, although the figures were drawn to correspond to the same chlorophyll concentration.

TABLE I

RELATIVE HEIGHTS OF FLUORESCENCE EMISSION BANDS AT −196° IN VARIOUS PARTICLE FRACTIONS OBTAINED BY DIGITONIN TREATMENT

Excitation light, see MATERIALS AND METHODS. Fraction numbers (f) correspond to those given in Fig. 1.

Particle fractions	*Fraction numbers (f)*	*F695/F684*	*F735/F684*	*F735/F680*
Chloroplasts		1.2	3.0	—
Digitonin-treated mixture		1.0	0.93	—
F-3	f-7	1.1	0.38	—
F-2	f-11	1.1	0.33	—
	f-12	1.0	0.32	—
	f-13	1.1	0.34	—
F-1	f-20	1.0	13	—
	f-21	1.0	14	—
	f-22	1.2	16	—
	f-23	0.73	8.0	—
	f-24	—	—	1.1
	f-25	—	—	4.5

Photochemical activities of the particle fractions

The activities of the Hill reaction with ferricyanide and the photoreduction of $NADP^+$ in the presence of ascorbate and 2,6-dichlorophenolindophenol as the electron donor system were measured in each fraction obtained by sucrose density gradient centrifugation. As shown in Fig. 3a, the localization of the Hill reaction activity

was in good agreement with the distribution of chlorophyll in F-2 and F-3. There was no Hill reaction activity in F-1. On the other hand, the photoreduction of $NADP^+$ was observed only in F-1 (Fig. 3). The relatively high activities of $NADP^+$ photoreduction in experiments shown in Fig. 3b are probably due to the improved conditions of assay performed with a reaction mixture containing equal amounts of chlorophyll. The levels of Hill reaction activities in the representative fractions of F-2 and F-3 (80 and 90 μmoles/mg chlorophyll per h) amounted to about 45% of that in the original chloroplasts (190 μmoles/mg chlorophyll per h). This decrease in activity must have been caused by the digitonin treatment employed here. The recovery during the fractionation by the density gradient centrifugation, however, was satisfactory, about 90% of the total activity in the detergent-treated mixture being discovered in F-2 and F-3.

The effects of the detergent treatment on the primary photochemical reaction of System II were examined by measuring the time-courses of fluorescence at 685 nm in various particle fractions. According to MURATA[13], the value for $f_t = F_s - F_0/F_s$, *i.e.* the ratio of the transient component of fluorescence ($F_s - F_0$) to the steady-state level of fluorescence (F_s), corresponds to the real quantum yield of photochemical Reaction II, which was adopted in the present experiment for comparing the primary photochemical activities of System II in various fractions. Fig. 4 shows the time-courses of fluorescence in the presence of 10 μM 3-(3',4'-dichlorophenyl)-1,1-dimethyl-

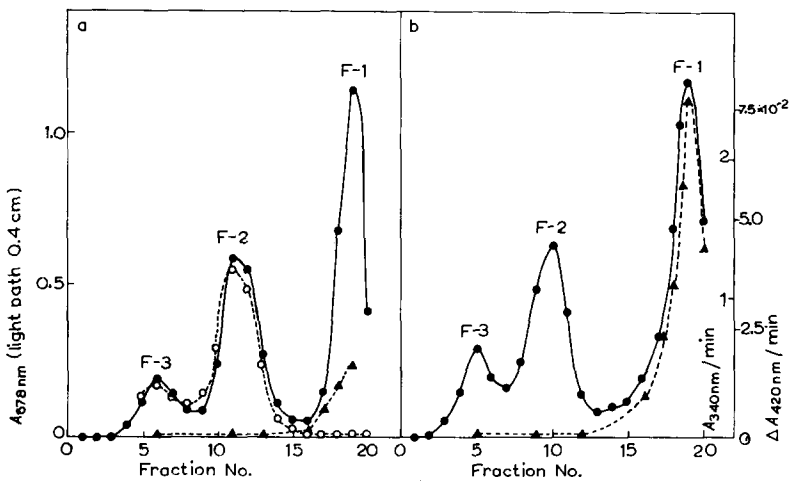

Fig. 3. Photoreductions of ferricyanide and $NADP^+$ in various fractions. Reaction mixture contained in 1 ml: particle fractions containing 4–6 μg chlorophyll; and, in μmoles, Tricine buffer (pH 7.3), 50; NaCl, 10; (for photoreduction of ferricyanide) ferricyanide, 0.4; methylamine·HCl, 15; (for photoreduction of $NADP^+$) $NADP^+$, 0.15; ascorbate, 3; 2,6-dichlorophenolindophenol, 0.03; DCMU, 0.01; saturating amounts of plastocyanin, ferredoxin and ferredoxin–$NADP^+$ reductase. Photoreduction of $NADP^+$ was measured anaerobically. Reaction time, 2 min. Photoreduction activities in each fraction were expressed by ΔA/min (with fractions having higher contents of chlorophyll, actual measurements were carried out after appropriate dilution). a. ●—●, absorbance at 678 nm; ○—○, $\Delta A_{420\,nm}$/min; ▲—▲, $\Delta A_{340\,nm}$/min. The distribution of chlorophyll in this set of experiments was: F-1, 51% (free chlorophyll, 9.5%); F-2, 34%; F-3, 14%. Hill reaction activity with ferricyanide: original chloroplasts, 190; digitonin-treated mixture, 48; F-2 (averages of f-10, f-11, f-12), 80; F-3 (f-5, f-6), 90. Photoreduction activity with $NADP^+$: digitonin-treated mixture, 40; F-1 (f-17, f-18, f-19), 140 (μmoles/mg chlorophyll per h). b. The distribution of chlorophyll was: F-1, 52%; F-2, 35%; F-3, 13%. Photoreduction activity with $NADP^+$ in F-1 (f-17–f-20), 470 (μmoles/mg chlorophyll per h).

urea (DCMU) at room temperature. The relative fluorescence yield in the steady state was significantly higher in F-2 and F-3 and markedly lower in F-1 (Fig. 4, Table II). The values for f_t in F-2 and F-3 were the same as that observed in the original chloro-

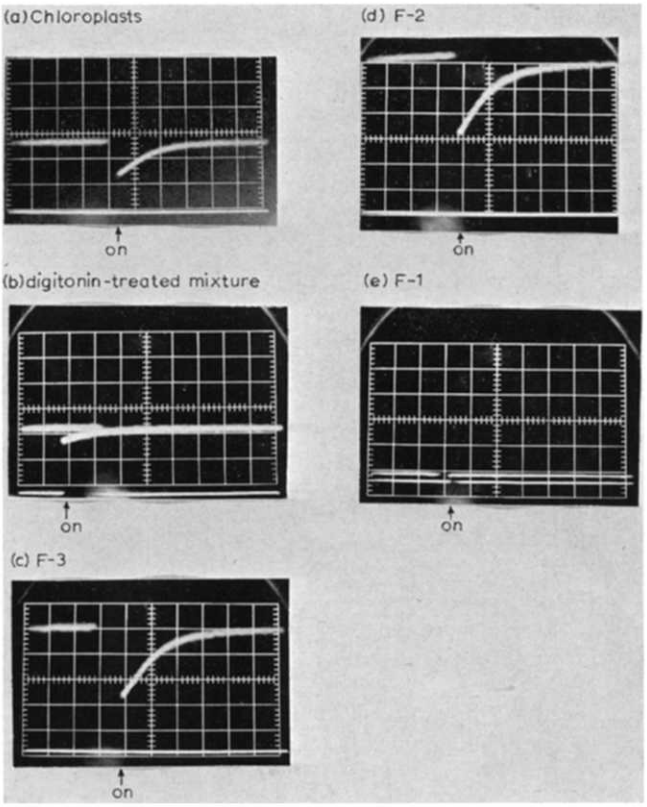

Fig. 4. Induction of fluorescence in various fractions. Synchroscope traces of fluorescence induction. One division corresponds to 1 sec. Reaction mixture contained 0.05 M Tricine buffer (pH 7.3), 1 mM $MgCl_2$, 10 μM DCMU and particle fractions. Absorbance at 435 nm of each sample in the measurements was: F-1, 0.1; F-2, 0.093; F-3, 0.1. Induction of fluorescence was measured at 685 nm at room temperature. The arrows indicate the onset of illumination. The straight line at the bottom of each figure corresponds to zero level of emission.

TABLE II

INDUCTION OF FLUORESCENCE IN VARIOUS FRACTIONS OBTAINED BY DIGITONIN TREATMENT

Experimental conditions, the same as in Fig. 4. Chlorophyll concentrations are expressed in absorbance at 435 nm. Values for F_0, F_s and F_s/chlorophyll are expressed in relative units.

Fractions	*Chlorophyll concn. (A at 435 nm)*	F_0	F_s	F_s/*chlorophyll*	$f_t = F_s - F_0/F_s$
Chloroplasts	0.12	15	29	24	0.48
Digitonin-treated mixture	0.13	21	25	19	0.18
F-3	0.1	22	48	48	0.55
F-2	0.093	31	63	67	0.50
F-1	0.1	3.5	3.5	3.5	0.00

plasts, thus showing that there was no significant impairment of the primary photochemical activity of System II due to the digitonin treatment. The low activity of Hill reaction in F-2 and F-3 described above, therefore, must be due to an impairment of some step(s) in the whole sequence of the Hill reaction. In marked contrast, no induction of fluorescence was observed in F-1, thus indicating complete absence of the primary photochemical activity of System II in this fraction.

DISCUSSION

The digitonin treatment was first used by WESSELS[3] and further extended by BOARDMAN AND ANDERSON[1]. The sucrose density gradient centrifugation was also used for a purification of System I particles by WESSELS[14] and for that of System II particles by HUZISIGE *et al.*[5].

In our present study, it was found that the digitonin treatment at a relatively high digitonin/chlorophyll ratio (20:1, w/w) and subsequent centrifugation on a linear sucrose density gradient provide a simple satisfactory method for the separation of the two particle units. Actually the whole green material was separated into three distinct bands, representing the photochemical System I (F-1) and the photochemical System II (F-2 and F-3). The F-2 and F-3 fractions showed essentially the same properties, so far as examined in the present experiments, except for the difference in the centrifugal behaviors described above. It was probably due to some difference in particle size or state of aggregation. No other fraction of intermediate nature was discovered so far as the green material was concerned.

The criteria used in this study for the assignment of the System I and System II particles were the molar ratio of chlorophyll *a*/chlorophyll *b*, the content of P700, the fluorescence emission spectrum at $-196°$, the photoreductions of ferricyanide and $NADP^+$, and the induction of fluorescence, criteria which have also been employed by previous investigators in various combinations[1,15–20]. The separation of the two systems obtained in the present study seems to be satisfactory, judged on standards of these criteria. The chlorophyll *a*/chlorophyll *b* ratios of 5.2 in F-1, 2.0 in F-2 and 2.2 in F-3 will be reasonable for assigning these particle fractions to Systems I and II. P700 was mainly localized in F-1. Only small amounts of total P700 (about 10%) were discovered in F-2 and F-3. The low-temperature fluorescence emission spectra also indicated the predominance of System I chlorophyll form (F735)[12,15] in F-1 and System II chlorophyll forms (F684, F695) in F-2 and F-3. Photochemical reaction activity of System I (photoreduction of $NADP^+$) was exclusively discovered in F-1 while that of System II (Hill reaction with ferricyanide) as well as its primary photochemical reaction II was exclusively distributed in F-2 and F-3.

The distribution of chlorophyll in System I (F-1) and System II (F-2 *plus* F-3) particles was about 40 and 53%, respectively. The amount of free chlorophyll was only 7% of the total chlorophyll in the detergent-treated mixture. The distribution of chlorophyll changed under various conditions of digitonin treatment; the higher the ratio of digitonin/chlorophyll, the more chlorophyll was recovered in F-1. At such high concentrations of digitonin (digitonin/chlorophyll, 30:1, w/w), the fluorescence analysis indicated that some portion of System II particles (or pigment System II chlorophyll *a*) was included as a contaminant in the lighter fraction F-1. The reverse was the case at lower ratios of digitonin/chlorophyll (for example, 12:1,

w/w); the amounts of chlorophyll in the heavier fractions F-2 and F-3 were higher and, moreover, there was an increase in the relative ratio of F735 to other System II fluorescence bands (F684, F695), thus indicating an incomplete separation of the two particles. A similar circumstance was encountered concerning the distribution of P700; on lowering the ratio, more P700 was discovered in the F-2 and F-3 fractions.

BOARDMAN *et al.*[15] and ANDERSON *et al.*[17], using the digitonin treatment have also reported the concentration of P700 and the predominance of F735 (low-temperature fluorescence emission) in their System I particles. It seems that the separation of System I particles was successful. However, as judged from their data of P700 content and fluorescence emission spectra, the degree of purity of their System II particles was rather low.

VERNON *et al.*[2] have obtained separation of System I and System II particles from spinach chloroplasts using relatively high concentrations of Triton X-100. However, Triton X-100 treatment inactivated the Hill reaction of System II particles and removed considerable amounts of chlorophyll from the lamellae of chloroplasts, as inferred from the fluorescence emission spectrum of their System I particles (PD-10 fraction)[16].

In the course of the present work, MICHEL AND MICHEL-WOLWERTZ[19] published a paper reporting separation of System I and System II particles by sucrose density gradient centrifugation. They used a French press for disintegration of the spinach chloroplasts. The possibility of disruption of lamellae in the two particle moieties by this purely mechanical treatment is of interest. However, judging from their data for the values of P700/chlorophyll, chlorophyll *a*/chlorophyll *b* and fluorescence emission spectra, the extent of separation of the two particles seems to be rather incomplete. Moreover, the photochemical activities reported for the isolated particle fractions seem to be low, indicating some impairment of the activities during the disruption-isolation procedures.

In summary, the results obtained in the present experiments furnish additional confirmative evidence for the assumption that the lamellae of the chloroplasts consist of System I and System II particles. The proportion of System I and System II particles in the lamellae of chloroplast was inferred to be about one to one on a chlorophyll basis, if we neglect the ambiguity due to the splitting of a small portion (less than 10%) of chlorophyll from the lamellar structure during the digitonin treatment.

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