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PHOTOCHEMICAL ACTIVITY AND STRUCTURAL STUDIES OF PHOTOSYSTEMS DERIVED FROM CHLOROPLAST GRANA AND STROMA LAMELLAE*

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SUMMARY

Stroma lamellae isolated by French press treatment, and Photosystem I and II fractions isolated by digitonin treatment of the grana fraction (free of stroma lamellae), were compared with respect to electron transport activities and ultrastructural characteristics. The results show:

1. About 15 % of the chlorophyll in mature spinach leaf chloroplasts occurred in the stroma lamellae. The separated grana membranes, when fractionated with digitonin, were comprised of 60 % Photosystem II and 40 % Photosystem I (on a chlorophyll basis), with essentially complete recovery of the starting material.

2. Action spectra and electron transport data for characteristic Photosystem I and II activities supported the concept that $NADP^+$ reduction activity was closely correlated with the content of P700 in the fractions. In no instance were there significant rates of $NADP^+$ reduction from water or an alternative Photosystem II donor (diphenylcarbazide) in the Photosystem II enriched fraction, beyond that which could be entirely accounted for by the residual P700 content.

3. The grana Photosystem II fraction, low in P700 and water or diphenyl carbazide $\rightarrow$ $NADP^+$ electron transport activity, could be recombined with the grana Photosystem I fraction and the reconstituted preparations were then capable of increased rates of diphenyl carbazide $\rightarrow$ $NADP^+$ reduction which were sensitive to a Photosystem II inhibitor, dichlorophenylmethylurea (DCMU). Stroma lamellae Photosystem I fraction, prepared by digitonin treatment was not capable of recombining with the grana Photosystem II fraction.

For the grana, the results are most simply explained by the series electron transport scheme which assumes that $NADP^+$ reduction from water normally proceeds *via* a series interaction between Photosystem II and a P700-containing Photosystem I. However, it cannot be unequivocally stated that the electrons from Photosystem II pass through P700 since none of our measurements followed P700 turnover directly.

Abbreviations: DCIP, dichlorophenolindophenol; DCMU, dichlorophenylmethylurea; Chl, chlorophyll.

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4. Photosystem I obtained from stroma and grana membranes were quite similar with regard to electron transport activity, P700 content, ultrastructure appearance and ultrafiltration characteristics, and differing in that stroma Photosystem I fragments did not recombine with the grana Photosystem II fraction and reconstitute electrons transport activity from diphenyl carbazide → $NADP^+$.

Plastocyanin was present in both grana and stroma membranes as shown by a sensitive bioassay.

5. The data are consistent with the chloroplast membrane model proposed earlier by ARNTZEN, DILLEY AND CRANE [*J. Cell. Biol.*, 43 (1969) 16] as regards the binary nature of the grana membranes, with Photosystem I localized on the outer "half" and Photosystem II on the inner "half" of a 90-Å grana thylakoid membrane.

INTRODUCTION

It is generally, but not universally, accepted that electron transport from water to $NADP^+$ in chloroplasts requires the participation of two photochemical steps arranged in series[1,2]. This concept was greatly strengthened by finding that two physically separable fractions could be isolated from chloroplasts treated with digitonin[3,4]. One fraction is enriched in the photoactive pigment, "P700", and is denoted Photosystem I. The photoact associated with this fraction produces a strong reductant capable of reducing electron acceptors with potentials as low as −650 mV (ref. 5), more than adequate to reduce $NADP^+$ ($E'_0 = -320$ mV). The fraction enriched in water oxidizing activity, Photosystem II, produces a weak reductant capable of reducing acceptors of potential about −35 mV or higher such as ferricyanide or dichlorophenolindophenol (DCIP)[6]. Thus, the Photosystem II enriched fraction as isolated by digitonin or Triton X-100 fractionation (see ref. 7 for a review of these results from several laboratories), is not capable of appreciable rates of $NADP^+$ reduction using either water or an electron donor for Photosystem II (such as diphenyl carbazide) due to the inability to form a strong reductant. There is usually some $NADP^+$ reduction activity left in the Photosystem II enriched fraction[4,7,8]. BOARDMAN[7] attributes the water → $NADP^+$ reduction activity in the Photosystem II fraction to residual contamination by Photosystem I, while ARNON and co-workers[8,9] believe that the water → $NADP^+$ activity in this fraction is an expression of $NADP^+$ reduction by Photosystem II which does not involve Photosystem I and P700 photochemistry. A single photoact was postulated by ARNON[9] to transfer an electron from water to $NADP^+$. This hypothesis was altered recently to include two photoacts, acting in series to accomplish $NADP^+$ reduction from water[10], but still it is postulated that P700 is not involved. According to this group, the P700 is involved only in cyclic phosphorylation.

RURAINSKI *et al.*[31] have also presented evidence that P700 and $NADP^+$ reduction activity (from water as an electron donor) are located in separate photosystems. Their experiments are based on the measurement of P700 turnover by an elegant relaxation technique. Assuming that their technique measures all the P700 turnover (*i.e.*, that none of the reaction centers are turning over so fast as to not be detected), their data showing a rise in $NADP^+$ reduction rate and a fall in P700 turnover rate with increasing $MgCl_2$ concentrations are strong evidence against the series scheme.

It is known from the work of JACOBI AND LEHMANN[11] and SANE *et al.*[12] that stroma lamellae contain Photosystem I with little or no Photosystem II and that grana lamellae contain both photosystems. These authors showed that one may readily separate the grana and stroma membranes by physical disruption followed by differential centrifugation. With these techniques available, coupled with the fact that digitonin gives a good separation of Photosystem I and Photosystem II activity with essentially 100 % recovery of the starting material, we felt that it was possible to critically test the hypothesis of ARNON *et al.*[10] and RURAINSKI *et al.*[31].

A critical test of this hypothesis includes starting with purified grana membranes free of stroma lamellae. This is essential since the stroma P700 will not then contaminate the P700 and grana Photosystem I obtained from fractionation. The next step involves digitonin fractionation of grana membranes followed by sucrose density gradient isolation of grana Photosystem I and II. Digitonin effectively separates Photosystem I and II while allowing nearly 100 % recovery of the starting materials. This is important because any conclusions regarding pathways of electron transport in intact grana, based on activities in solubilized subfractions, will be greatly strengthened by recovering all the chlorophyll containing membranes with partial electron transport activity maintained. If the ARNON *et al.*[10] hypothesis is correct, one should recover a significant amount of the grana in a fraction having all or most of the water $\rightarrow$ $NADP^+$ electron transport activity but very little of the P700. In the more simple view, a null test of this hypothesis would result from finding a Photosystem II fraction having most of the DCMU inhibitable water or diphenyl carbazide $\rightarrow$ DCIP activity, very little P700 and very little ascorbate + DCIP $\rightarrow$ $NADP^+$ activity, the latter activity being recovered in a separate fraction, having little Photosystem II activity. We will show just this type of result, indicating that grana Photosystem I (the P700 reaction center) normally is required to interact with Photosystem II to give water or diphenyl carbazide $\rightarrow$ $NADP^+$ electron transport activity.

As a further indication that Photosystem I and II interact in this way, we will show reconstitution of diphenyl carbazide $\rightarrow$ $NADP^+$ activity when grana Photosystem I particles are added back to the Photosystem II fraction in the presence of exogenous lipid.

METHODS

Preparation of grana and stroma lamellae

Spinach chloroplasts were isolated in 0.5 M sucrose, 0.05 M K_2HPO_4 buffer (pH 7.4) and 0.01 M KCl (ref. 12). The pellet was resuspended to 0.5 mg chlorophyll (Chl)/ml in 0.15 M KCl containing 0.05 M K_2HPO_4 buffer (pH 7.4) and subjected to French press treatment at 4000 lb/inch^2 pressure as previously described[12,18]. The flow sheet in Fig. 1 shows the details of French press treatment and centrifugations used to isolate stroma lamellae from grana. It should be noted that we collect only a 40000 $\times$ *g* fraction rather than 1000, 10000 and 40000 $\times$ *g* fractions as did SANE *et al.*[12]. This allows a straight forward determination of the amount of Photosystem I particles released by treatment since the 40K (see Fig. 1) supernatant (which contains only Photosystem I) can be assayed directly for chlorophyll content and this value can be compared to the initial concentrations during pressure treatment.

Digitonin fractionation

The $40K_3$ (see Fig. 1) grana fraction was resuspended in 0.01 M KCl, 0.05 M K_2HPO_4 buffer (pH7.2) to give a chlorophyll concentration of 0.25 mg Chl/ml. Three parts of this suspension were added to 1 part 2.0 % digitonin (twice recrystallized and thoroughly vacuum desiccated to remove traces of ethanol). This mixture (containing a final concentration of 0.5 % digitonin) was incubated at 15° for 30 min with constant stirring. After this period, 5 ml of the mixture were layered into sucrose density gradient tubes having the following bands: bottom, 15 ml 1.8 M sucrose; middle band, 10 ml 1.0 M sucrose; top, 9.0 ml 0.7 M sucrose. All sucrose solutions were made up in 0.05 M phosphate buffer (pH 7.2). Centrifugation time was 60 min at 27000 rev./min in a Spinco SW-27 rotor.

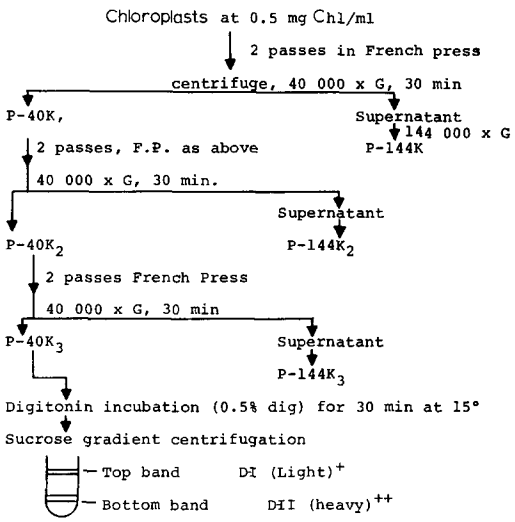

Fig. 1. Preparation diagram of grana and stroma membrane fractions. The spinach chloroplasts were prepared and handled as described in METHODS.

Following centrifugation, a broad band of chlorophyll extended from the top of the tube to the 0.7–1.0 M sucrose interface. This was removed by removing the top 12 ml of the tube contents with a flat-tipped syringe needle (Fraction D-I). A second aliquot of 10 ml was then removed and designated as Fraction D-II. This included a sharp, heavy band of chlorophyll which collected at the 1.0–1.8 M sucrose interface.

The bottom band contained primarily Photosystem II activity and the top band primarily Photosystem I as shown earlier by OHKI AND TAKAMIYA[19] who used a linear rather than a step gradient. In some instances, for better separation of Photosystem I from the Photosystem II fraction, we included 0.5 % digitonin in the top sucrose band, so that the heavy Photosystem II membrane pieces would tend to lose as much Photosystem I as possible while going through the sucrose.

For some studies we collected Photosystem II and I fractions by differential centrifugation according to earlier workers[4]. In these cases the pellets were resuspended in 0.4 M sorbitol, 0.01 M KCl and 0.02 M Tricine buffer (pH 7.8).

Ultrafiltration of membrane fractions

To assess the degree of aggregation and/or the particle size of the Photosystem I and II fractions, we have used Amicon and Sartorius ultrafilters with various pore sizes. Filter pore sizes used were: Sartorius 500 A (S-113 10 47) and 100 A (S-113 11 47); Amicon 120 A (XM 100 A), and 65 A (XM 50). An Amicon ultrafiltration cell operated at 10 lb/inch^2 pressure with constant stirring at 0° was used for all experiments.

Fig. 2 shows a flow sheet illustrating the procedure used to test the relative size of the Photosystem I fraction derived from grana, stroma and whole chloroplasts. In these experiments the supernatant from a 40000 × *g* centrifugation was applied to the filters. The Photosystem I and II activities of the initial samples and of the filtrate were measured. A comparison of the chlorophyll concentration in the initial solution and the filtrate allowed a determination of the percent of the sample retained by each of the filters.

Aliquots of certain filtrates were taken for electron microscopy.

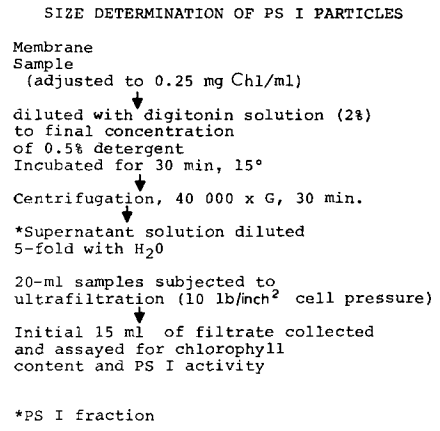

Fig. 2. Protocol for ultrafiltration experiments using Amicon–Diaflo or Sartorius filters. See text for details. PS I, Photosystem I.

Photosystem I and II reconstitution studies

The isolated Photosystem I and II fractions were recombined under various conditions and assayed for electron transport activity from diphenyl carbazide to $NADP^+$. The appropriate table or figure legends give specific details. In general, it was necessary to combine Photosystem I and II fractions as concentrated as possible rather than dilute to obtain the highest electron transport rates. Asolectin (a soybean lipid extract) or purified lecithin (Sigma Grade II-S) were added to the concentrated Photosystem I and II mixture to obtain high rates of diphenyl carbazide→$NADP^+$ activity. Purified plastocyanin, ferredoxin and ferredoxin–$NADP^+$ reductase or a crude enzyme preparation containing all three enzymes were added to the reconstituted particles before diluting with buffer for the assay.

Since the oxidation of diphenyl carbazide gives rise to an increase in absorbance at 340 nm (ref. 21), the ΔA measured at 340 nm was corrected by multiplying by 0.66, the following ratio of extinction coefficients of oxidized diphenyl carbazide to NADPH, $\varepsilon_{NADPH}/[\varepsilon_{NADPH} + \varepsilon_{DPC}]$.

Electron transport and other assays

Chlorophyll determination and Chl *a*/*b* ratios were carried out as described by ARNON[20]. Photosystem II electron transport was assayed by following DCIP reduction spectrophotometrically (at 580 nm) using diphenyl carbazide as the electron donor substituting for water[21]. With Photosystem II particles, the diphenyl carbazide→ DCIP activity is DCMU inhibited at the usual concentrations, as expected for a Photosystem II reaction. To be certain, we checked the DCMU effect in most assays. Photosystem I activity was assayed by following $NADP^+$ reduction (at 340 nm) using ascorbate + DCIP as the electron donor system. Purified plastocyanin, ferredoxin, and ferredoxin–$NADP^+$ reductase were added in saturating amounts. In some cases, as indicated, low amounts of Triton X-100 were added in order to obtain maximum rates of the reaction. Triton X-100 probably facilitates the entry of plastocyanin into the membrane. See the legends for concentrations of reaction components. In some cases, Photosystem I activity was followed using ascorbate + DCIP→ methyl viologen and measuring O_2 uptake[22]. Saturating amounts of plastocyanin were added in such assays.

Action spectra of diphenyl carbazide→ DCIP reduction (a Photosystem II activity) and ascorbate + DCIP→ $NADP^+$ reduction (a Photosystem I activity) were performed with Baird Atomic interference filters having about 5 nm half-band widths. The light intensities used for sample illumination were checked with a Kettering YSI light meter which reads directly in ergs/cm^2 per sec. The data were obtained at constant light intensity at all wavelengths. The intensity was below saturation for any wavelength. The spectrophotometer used to follow the rate of the reaction was a modified Bausch and Lomb 505, fitted with an entrance port for actinic light at right angles to the measuring beam. The entire reaction mixture was illuminated. For plotting the action spectra, the rate of the reaction at each wavelength was divided by the wavelength to correct for the difference in the number of quanta per unit of energy at different wavelengths.

Plastocyanin bioassay

To measure the plastocyanin content of the various membrane fractions obtained, we employed a bioassay similar to that recently published by PLESNICAR AND BENDALL[26]. The bioassay consisted of sonicating the membrane sample 10 min to release the plastocyanin[25], and using that supernatant (after 1 h of centrifugation at 144000 × *g* to remove the membrane material) as part of the reaction mixture in an ascorbate + DCIP→ $NADP^+$ assay in digitonin Photosystem I particles which require added plastocyanin for activity. A standard curve was prepared by measuring $NADP^+$ reduction due to the addition of known amounts of purified plastocyanin.

RESULTS

Isolation of Photosystem I and II

Since it was imperative for this study to obtain clean preparations of grana and stroma lamellae for comparison and characterization studies, the extensive preparative procedure outlined in Fig. 1 was adopted. We had earlier shown[18] that two passes through the French pressure cell at 4000 lb/inch^2 pressure gave optimal release of the light stroma lamellae. It was found in this study that six French pressure treat-

TABLE I

PHOTOSYSTEM I AND II DERIVED FROM GRANA, AND PHOTOSYSTEM I DERIVED FROM STROMA MEMBRANES

Grana and stroma lamellae were separated and treated with digitonin as shown in the flow sheet Fig. 1. The chloroplast suspension media were given under METHODS, as were the assays. The precision of the P700 assay was about ± 20%. The Photosystem II assay media contained: 0.5 mM diphenyl carbazide, 0.1 mM DCIP, 50 mM K_2HPO_4 buffer (pH 6.8) and 20 μg Chl per ml in a total volume of 2.0 ml. The Photosystem I assay media contained: 50 mM K_2HPO_4 buffer (pH 6.8), 7.5 mM sodium ascorbate, 0.16 mM DCIP, 0.37 mM $NADP^+$, 20 μg Chl per ml. 1.3 μM of plastocyanin, and saturating amounts of spinach ferredoxin, and ferredoxin–NADP reductase in a total volume of 2 ml. The light intensity incident on the cuvette for enzymatic assays was $2 \cdot 10^5$ ergs/cm² per sec. Red light was used by employing a Corning No. 2403 red filter and a No. 4600 infrared filter. The protocol for the spectrophotometric assays is found in ref. 36. For the P700 assays, an absorbance coefficient of 65 $mM^{-1} \cdot cm^{-1}$ was used, a value recently determined by T. HIYAMA AND B. KE (Personal Communication).

Fraction	*Recovery of Chl (%)*	*Photosystem II* activity*	*Photosystem I** activity*	*Chl/P700 ratio*	*Chl a/b ratio*
Chloroplasts	100	440	465	340	2.8
P-40K_3	86	125	240	460	2.2
D-I	35.0	8	490	170	3.7
D-II	51.0	122	43	1730	1.8
	86.0				
P-144K_1	9.5	3	754	100	5.6
P-144K_2	3.0	3	800	120	5.3
P-144K_3	1.5	9	503	141	4.4
	100.0				

* Assayed by diphenyl carbazide → DCIP (DCMU-sensitive portion).

** Assayed by ascorbate + $DCIPH_2$ → $NADP^+$ (Triton X-100, plastocyanin, ferredoxin, and ferredoxin–$NADP^+$ reductase).

ments of the grana fraction (Fraction P-40K) were necessary to release essentially all of the stroma membranes. As is shown in Table I, the recovery of stroma lamellae (Fractions P-144K) was as high as 9.5 % after one pressure treatment but dropped to 3 % and then 1.5 % in subsequent treatments. Preliminary work revealed that a fourth treatment yielded less than 1 % stroma lamellae. It was therefore assumed that Fraction P-40K_3 could be designated as grana membrane, with only a very minor contamination of stroma membranes.

The grana membranes were treated with digitonin as previously described[4,14], except that the digitonin chloroplast mixture was maintained at 15° instead of 0–2°. Preliminary experiments had shown that this procedure gave somewhat more complete separation of grana Photosystem I and II, without significant decrease of photochemical activities. Since it was important for this study to make accurate quantitative determinations of the amount of grana Photosystem I and II obtained, a sucrose gradient procedure modified from that of OHKI AND TAKAMIYA[19] was used rather than a differential centrifugation procedure to isolate the two fractions. The two bands (D-I and D-II) from the gradient were assayed for chlorophyll content and recovery values were calculated directly. Table I shows that the Fractions D-II and D-I comprise about 60 and 40 % of the grana membranes, respectively.

Biochemical characterization of membrane fractions

The P700 content, photochemical activities, and Chl *a*/*b* ratios of the various membrane fractions are also shown in Table I. Several critical points of interest about these data and the other information shown in Table I are: (a) From the original whole chloroplasts, a total of 14 % of the chlorophyll can be recovered in a stroma lamellae fraction (P-144K_{1-3}) following French pressure treatment. The 86 % chlorophyll recovered in grana membranes (P-40K_3) can further be broken down by digitonin treatment to give Photosystem I (D-I) and Photosystem II (D-II) fractions which represent 35 and 51 % of the original chloroplast chlorophyll, respectively. (b) As has been shown previously[12], the stroma lamellae are deficient in Photosystem II activity, enriched in P700 content, and have higher Chl *a*/*b* ratios than control chloroplasts. The reduced electron transport rates of the grana fraction with relation to whole chloroplasts is probably the result of the extensive isolation procedure. (c) Digitonin treatment of grana membranes allowed the isolation of purified Photosystem I (D-I) and greatly enriched Photosystem II (D-II) fractions. The grana Photosystem I sample had higher P700 content and Chl *a*/*b* than control chloroplasts, although the values were not as high as for the stroma lamellae fractions. The Photosystem II fraction had a greatly reduced P700 content and a very low Chl *a*/*b* ratio. As a check on our chemical method of assaying for P700, we also measured the P700 content in similar fractions by the photochemical oxidation of P700 (assays kindly carried out by Dr. B. C. Mayne). The values so obtained were in quite good agreement, grana Photosystem II (D-II) 1390 Chl/P700; grana Photosystem I (D-I) 312; and intact grana, 517.

The plastocyanin content of stroma lamellae, grana membranes and intact chloroplasts has also been determined. The stroma particles were found to contain plastocyanin at a ratio of 1 plastocyanin per 1500 chlorophylls (Table II). The grana fraction contained 1 plastocyanin per 2200 chlorophylls, and control chloroplasts contained 1 plastocyanin per 500 chlorophylls. About 40 % of the plastocyanin in control chloroplasts is lost to the suspending medium during the French press treatment and subsequent centrifugation as shown by line 6 of Table II.

Action spectra

As a further means of characterizing the various activities which we have used to assay either Photosystem I or II, the action spectra for these activities were determined for several fractions. Fig. 3 shows the results of action spectra measurements for $DCIPH_2 \rightarrow NADP^+$ and water $\rightarrow NADP^+$ activities in control chloroplasts, diphenyl carbazide $\rightarrow$ DCIP activity in the grana Photosystem II fraction (D-II), and $DCIPH_2 \rightarrow NADP^+$ activity in the stroma lamellae (P-144K_3) and grana light fraction (D-I). Water $\rightarrow NADP^+$ and diphenyl carbazide $\rightarrow$ DCIP both show typical Photosystem II action spectra, as expected since both activities require and are controlled by the short wavelength system. In all three cases, the $DCIPH_2 \rightarrow NADP^+$ activities have a definite red shift, both in the peak of the activity (685 nm compared to 680 nm for the Photosystem II activity), and in the level of activity at wavelengths greater than 685 nm.

Reconstitution of Photosystem I and II particles

If $NADP^+$ reduction using either water or diphenyl carbazide as the electron donor requires the participation of both Photosystem II and I, and given that digi-

TABLE II

PLASTOCYANIN CONTENT OF GRANA AND STROMA

The bioassay consisted of sonicating the membrane sample 10 min to release the plastocyanin[25,26] and using that supernatant (after 1 h of centrifugation at 144000 × *g* to remove the membrane material) as part of the reaction mixture in an ascorbate + DCIP → $NADP^+$ assay in digitonin Photosystem I particles which require added plastocyanin for activity. A standard curve was prepared by measuring $NADP^+$ reduction due to the addition of known amounts of purified plastocyanin. Aliquots of the supernatants were added to digitonin-derived Photosystem I particles in ascorbate + DCIP → $NADP^+$ assays (as in Table I) which had no other source of plastocyanin. The Photosystem I particles used showed no $NADP^+$ reduction without added plastocyanin. The amount of plastocyanin in the supernatant was estimated from a standard curve of rate *vs.* added plastocyanin.

Sample	*Electron transport rate (μmoles $NADP^+$ per h per mg Chl)*	*Plastocyanin in reaction mixture (nmoles)*	*Plastocyanin calculated to be in total sonicate (nmoles)*	*Chl††/ plastocyanin ratio*
1. Photosystem I	0	0	—	—
2. Photosystem I + purified plastocyanin (0.3 nmole/ml)	200	0.3	—	—
3. Photosystem I + whole chloroplasts extract (0.5 ml)	240	0.38*	9.5	500
4. Photosystem I + grana extract (0.5 ml)	74	0.080**	0.80	2200
5. Photosystem I + stroma extract (1.0 ml)	75	0.083***	0.535	1520
6. Photosystem I + 144 K supernatant (0.5 ml)	131	0.22†	465	1100

* 0.5 ml of 12 ml total supernatant from sonication of chloroplasts containing 4.75 mg Chl.
** 0.5 ml of 5 ml total supernatant from sonication of grana membranes having 1.10 mg Chl.
*** 1.0 ml of 6.4 ml total supernatant from sonication of stroma membranes containing 0.805 mg Chl.
† 0.5 ml of 1055 ml total volume of the 144000 × *g* supernatant from a French press stroma preparation having 528 mg Chl total in the original material.
†† The chlorophyll referred to here is from the chloroplasts from which plastocyanin was removed by sonication.

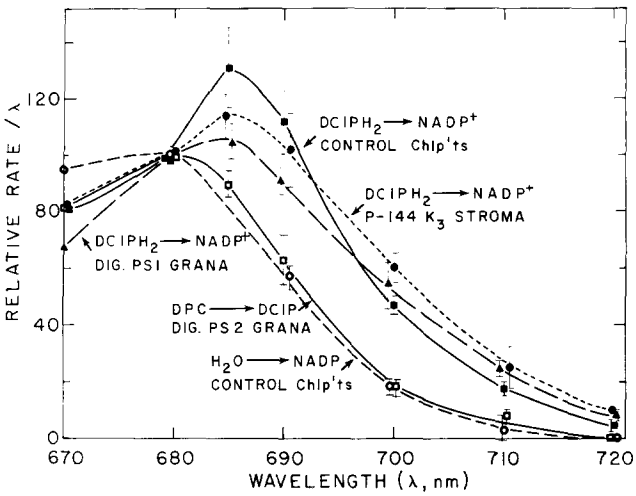

Fig. 3. Action spectra of electron transport in whole chloroplasts and Photosystem I and II fractions. The reaction conditions for the electron transport assays were as given in Table I. The rates were normalized to 100% at 680 nm and expressed as percent rates at other wavelengths. For all assays the light intensity incident on the cuvette was 10^4 ergs/cm^2 per sec^1. At wavelengths 700, 710 and 720 nm, a Corning 4-77 cut-off filter was used in addition to the Baird Atomic interference filter. This filter blocks 95% of 680-nm radiation and 100% in the range 600–670 nm, thus cutting off virtually all the radiation <680 nm which may be transmitted in the tail of the transmission curve for the interference filters. DPC, diphenyl carbazide.

tonin separates these two entities, then it should be possible to reconstitute the activity by recombining the Photosystem II and I fractions. In all reconstitution studies, we have used diphenyl carbazide as an electron donor for Photosystem II since the extensive isolation procedures inactivated the major portion of the water oxidizing capacity. We have carried out recombination experiments[27] and can indeed show a stimulation in NADP$^+$ reduction from diphenyl carbazide by mixing grana Photosystem I and II (D-I and D-II) fractions. Table III shows some typical data. Expt. A shows that Photosystem II alone gave a rate of 15 μmoles NADP$^+$/h per mg Chl and Photosystem II + grana Photosystem I gave a rate of 65 all of which was DCMU sensitive (indicative that Photosystem II was involved in the activity). A similar experiment with different chloroplast material gave rates of NADP$^+$ reduction from Photosystem II alone + lecithin of 10 μmoles/h per mg Chl and with Photosystem II + grana Photosystem I + lecithin 36 μmoles/h per mg Chl (Part C). Grana Photosystem I alone had no diphenyl carbazide → NADP$^+$ activity. Leaving out NADP$^+$ resulted in no ΔA, indicative that NADP$^+$ was acting as the electron acceptor. Optimal rates of diphenyl carbazide → NADP$^+$ activity required the presence of either asolectin or lecithin (Expt. B, Table III). The best results were found when Photosystem II, lecithin and grana Photosystem I were added together concentrated, followed by dilution. There was a variable stimulation in diphenyl carbazide → NADP$^+$ activity in the Photosystem II fraction due to addition of lecithin alone (Expt. B Lines 5 and 6). However, there was routinely 2-fold or greater additional stimulation when grana Photosystem I was added. Lecithin did not stimulate diphenyl carbazide → DCIP activity (Expt. D). If the grana Photosystem I was heated at 70° for 5 min before addition to Photosystem II, there was no stimulation.

TABLE III

RECONSTITUTION OF DIPHENYL CARBAZIDE → $NADP^+$ ACTIVITY BY COMBINING GRANA PHOTOSYSTEM I AND II FRACTIONS

Photosystem II fractions were prepared by digitonin incubation followed by sucrose density gradient centrifugations as discussed under METHODS. The Photosystem I fractions were prepared by the same method as the Photosystem II or by the differential centrifugation technique. The assays for diphenyl carbazide → $NADP^+$ activity were carried out as follows: Photosystem II fragments equivalent to 30 μg Chl (in about 10 μl) were mixed with 1.25 mg (in 50 μl) sonicated lecithin (Sigma) or asolectin (Associated Concentrates, Woodside, L.I., N.Y.) and Photosystem I fragments equivalent to 15 μg Chl (about 5 μl). After gentle shaking for 20–30 sec, the balance of the reaction mixture was added: 50 mM K_2HPO_4 buffer (pH 6.8), 1 mM diphenyl carbazide, 0.37 mM $NADP^+$, 1.3 μM plastocyanin and saturating amounts of ferredoxin and ferredoxin–$NADP^+$ reductase in a total volume of 2 ml. $NADP^+$ reduction was measured by following ΔA at 340 nm. Since the oxidation of diphenyl carbazide gives some increase in absorbance at 340 nm (ref. 21), this factor was corrected out of the total absorbance change by multiplying the observed ΔA by 0.66.

Additions		*Rate* (*μmoles $NADP^+$ per h per mg Chl*)
A. 1. Photosystem II + Photosystem I + lecithin		65
2. As No. 1 less Photosystem I		15
3. As No. 1 less Photosystem II		0
4. As No. 1 + 10 μM DCMU		0
5. As No. 1 less $NADP^+$		0
B. *Added concentrated*	*Added after dilution*	
1. Photosystem II + lecithin + Photosystem I	—	38
2. Photosystem II + Photosystem I	Lecithin	31
3. Photosystem II + lecithin	Photosystem I	33
4. Photosystem I + lecithin	Photosystem II	9.9
5. Photosystem II + lecithin	(Photosystem I omitted)	23
6. Photosystem II	(Photosystem I and lecithin omitted)	14
7. Photosystem II + Photosystem I	(Lecithin omitted)	24
C. 1. Photosystem II + lecithin + Photosystem I		36
2. Photosystem II + lecithin + (Photosystem I heated to 70° for 5 min)		11
Diphenyl carbazide → DCIP		*μmoles DCIP per h per mg Chl*
D. 1. Photosystem II		98
2. Photosystem II + lecithin		100

As a further aspect of the comparative study of grana and stroma membranes, it was of interest to test the relative effectiveness of stroma lamellae Photosystem I particles in reconstituting complete electron transport with grana Photosystem II fractions. For this purpose, stroma lamellae were treated with detergent under the same conditions as were grana membranes, and then concentrated by differential centrifugation. Since this differential centrifugation gave two fractions, both of which had Photosystem I activity (see Table IV, Part B), both fractions were tested for reconstitution capacity. Table IV (Part A) shows that neither stroma lamellae Photosystem I preparation was capable of interacting with the grana Photosystem II to support increased electron transport from diphenyl carbazide to $NADP^+$. However,

TABLE IV

COMPARATIVE RECONSTITUTION OF ELECTRON TRANSPORT USING GRANA OR STROMA PHOTOSYSTEM I FRAGMENTS WITH GRANA PHOTOSYSTEM II FRAGMENTS

Fractions were prepared by digitonin treatment of either grana or stroma lamellae. The conditions for reconstitution assays in Expt. A were as given in Table III. The grana Photosystem I and II (D-I and D-II) fractions were prepared as described in Fig. 1. Stroma lamellae (P-144K_1) were resuspended and incubated with digitonin under the same conditions as described for the grana fractions. Differential centrifugation of the stroma membrane-detergent suspension (40000 × *g* for 30 min and 144000 × *g* for 1 h) gave two stroma lamellae Photosystem I fractions. Electron transport assay conditions in Expt. B were as described in Table I.

Fractions added	*$NADP^+$ reduction rate (μmoles/h per mg Chl)*
A. 1. Photosystem II	19
2. Photosystem II + grana Photosystem I	42
3. Photosystem II + stroma Photosystem I (144000 × *g* pellet)	14
4. Photosystem II + stroma (40000 × *g* pellet)	15

	Photosystem I assay (ascorbate + DCIP → $NADP^+$) (μmoles $NADP^+$ per h per mg Chl)	*Photosystem II assay (diphenyl carbazide → DCIP) (μmoles DCIP per h per mg Chl)*
B. 1. Photosystem I from grana	1010	47
2. Photosystem II from grana	47	415
3. Photosystem I from stroma (144000 × *g* pellet)	805	21
4. Photosystem I from stroma (40000 × *g* pellet)	750	20

grana Photosystem I preparations from the same chloroplasts did give better than a 2-fold increase in $NADP^+$ reduction rate, in agreement with data shown in Table III.

Structural characterization of Photosystem I and II

It has been well established by this and preceding studies that mechanical treatment of chloroplasts causes the release of stroma lamellae which have only Photosystem I activity[11-13,18]. In addition, the data in Table I indicate that isolated grana membranes can be separated into Photosystem I and II components by detergent treatment. It was therefore of interest to conduct comparative structural studies of the various fractions. Our first approach to this problem was to use the ultrafiltration procedure for estimation of the particle size in each of the preparations. It should be pointed out that the exclusion limit for macromolecules by these filters is not extremely sharp, especially when the macromolecule is not a rigid sphere, and the retention of material by each of the filters gives only an approximation of the actual particle size of the sample being tested.

Whole chloroplasts and French press derived grana membrane and stroma lamellae fractions did not pass through any of the filters used in this study (see No. 1, Table V). In addition, a Photosystem II preparation prepared by detergent treatment of either whole chloroplasts or grana membranes (see No. 2, Table V) was completely retained by all the filters used. Both these findings were to be expected since all of these fractions had previously been described as membranous stacks or vesicles[12,14] which would not penetrate the small pores of the filter.

TABLE V

RETENTION OF VARIOUS CHLOROPLAST MEMBRANE FRAGMENTS ON ULTRAFILTERS

Whole chloroplasts, grana membranes (P-40K_3) and stroma lamellae (P-144K) were digested with digitonin and centrifuged as shown in Fig. 2 before being applied to the filters. The Photosystem I activity given is that prior to filtration, and was assayed by the ascorbate + DCIP → methylviologen assay. Reaction conditions were as described for $NADP^+$ reduction in Table I except ferredoxin, reductase, and $NADP^+$ were omitted, and 0.5 mM methylviologen and 0.4 M NaN_3 were added. The various Sartorius (Sart) or Amicon (Am) filters used are listed with the diameter of the pore sizes. Sample 1 was a stroma (P-144) preparation not treated with digitonin. Sample 2 was a Photosystem II sample derived from digitonin digestion and sucrose-gradient centrifugation. Sample 6 was the filtrate from the 120-Å Amicon filter reaggregated in salt and centrifuged, as explained in RESULTS, prior to being loaded back into a 500- or 100-Å filter.

Membrane sample	*Chl recovered in Photosystem I fraction after centrifugation* (%)	*Photosystem I activity of sample* (*μmoles methyl-viologen reduced per mg Chl per h*)	*Chl concn. in filter chamber fraction* (*μg/ml*)	*Retention* (%)			
				Pore diameter of filter:			
				500 Å (Sart)	*120 Å (Am)*	*100 Å (Sart)*	*65 Å (Am)*
1. Whole chloroplasts, grana membranes, or untreated stroma membranes	—	—	—	99–100% retention on all filters			
2. Photosystem II fraction by digitonin from sucrose gradient	—	—	—	99–100% retention on all filters			
3. Whole chloroplasts *	35	3400	16	38	63	43	99
4. Stroma (P-144K) *	67	6600	25	37	66	85	99
5. Grana (P-40K_3) *	47	3750	18	27	66	52	99
6. Grana Photosystem I (reaggregated) from Am-120 A filter	—	2500	4	96	—	97	—

* 40K supernatant after digitonin digestion.

Photosystem I fractions prepared by detergent treatment gave very different results. Digitonin incubation was used to solubilize the Photosystem I portion of whole chloroplasts, grana membranes, and stroma lamellae according to the protocol described in Fig. 2. The supernatant of the 40000 × *g* centrifugation in this procedure contained essentially no Photosystem II activity in any of the three preparations. The chlorophyll content and Photosystem I activity of the 40000 × *g* supernatant for each sample is shown in Table V (Items 3, 4 and 5), and the amount of chlorophyll retained by each of the filters is indicated. Approx. 2/3 of the Photosystem I material in all preparations easily passes through the 500-Å pore size filter, and about 1/3 to 1/2 of the Photosystem I chlorophyll in each case can penetrate a 100–120-Å pore size filter. All samples, however, were completely retained by the 65-Å pore size filter.

Two lines of evidence support the concept that the chlorophyllous material which passed through the 100- and 120-Å ultrafilters was actually associated with a Photosystem I reaction center particle rather than existing as solubilized chlorophyll. First, the Photosystem I activity of the fractions was high as compared with whole chloroplasts both before and after passage through the filter. Less than 10 % activity (either methyl viologen or $NADP^+$ reduction) was lost during filtration. Second, almost all chlorophyll was retained by a 65-Å pore size filter; this filter would not stop solubilized chlorophyll.

The previous structural studies on digitonin-derived membrane fractions[14] had shown that Photosystem I fractions do not always appear as particles. In particular, Figs. 9 and 10 of ref. 14 showed that Photosystem I particles purified by column chromatography in the presence of high salt existed in membranous vesicles. To test whether the isolation conditions has some effect on the structural organization of the Photosystem I particles, we extended the ultrafiltration study described above by treating the grana Photosystem I particles which were in the filtrate after passage through an Amicon 120-Å filter with salt. Concentrated salt solutions were added to the filtered Photosystem I preparation to give a final concentration of 0.1 M KCl, 0.05 M K_2HPO_4 buffer (pH 7.8) and 5 mM $MgCl_2$. This was mixed thoroughly and then centrifuged for 1 h at 144000 × *g*. The pellet from this centrifugation was resuspended in a salt solution of the same concentration and then reapplied to the Sartorius 500- and 100-Å filters (see No. 6 of Table V). In both cases, the chlorophyll was almost completely retained by the filter. As will be shown below, the high retention indicates that the small grana Photosystem I particles had reaggregated into a large membranous sheet or vesicle in the presence of high salt.

Negative staining and electron microscopic investigation give complete support to the ultrafilter results. Fig. 4 shows by negative staining, the grana Photosystem I material which passed through the 100-Å filter. The micrograph shows strands of material about 50 Å wide by 500 Å long (there is considerable variation in the strand lengths). This fraction passing through the filter retains Photosystem I activity. The appearance of these digitonin-derived Photosystem I particles is virtually identical to the Photosystem I fraction isolated from chloroplasts by Triton X-100 solubilization[23]. Stroma lamellae Photosystem I which had passed through a 100-Å filter showed essentially the same string-like structures. In some cases, the stroma particles demonstrated a greater tendency for aggregation into membranous sheets. A sample of such an aggregated stroma lamellae Photosystem I fraction is shown in Fig. 5.

When the fraction shown in Fig. 4 was suspended in 0.1 M KCl, 0.05 M K_2HPO_4

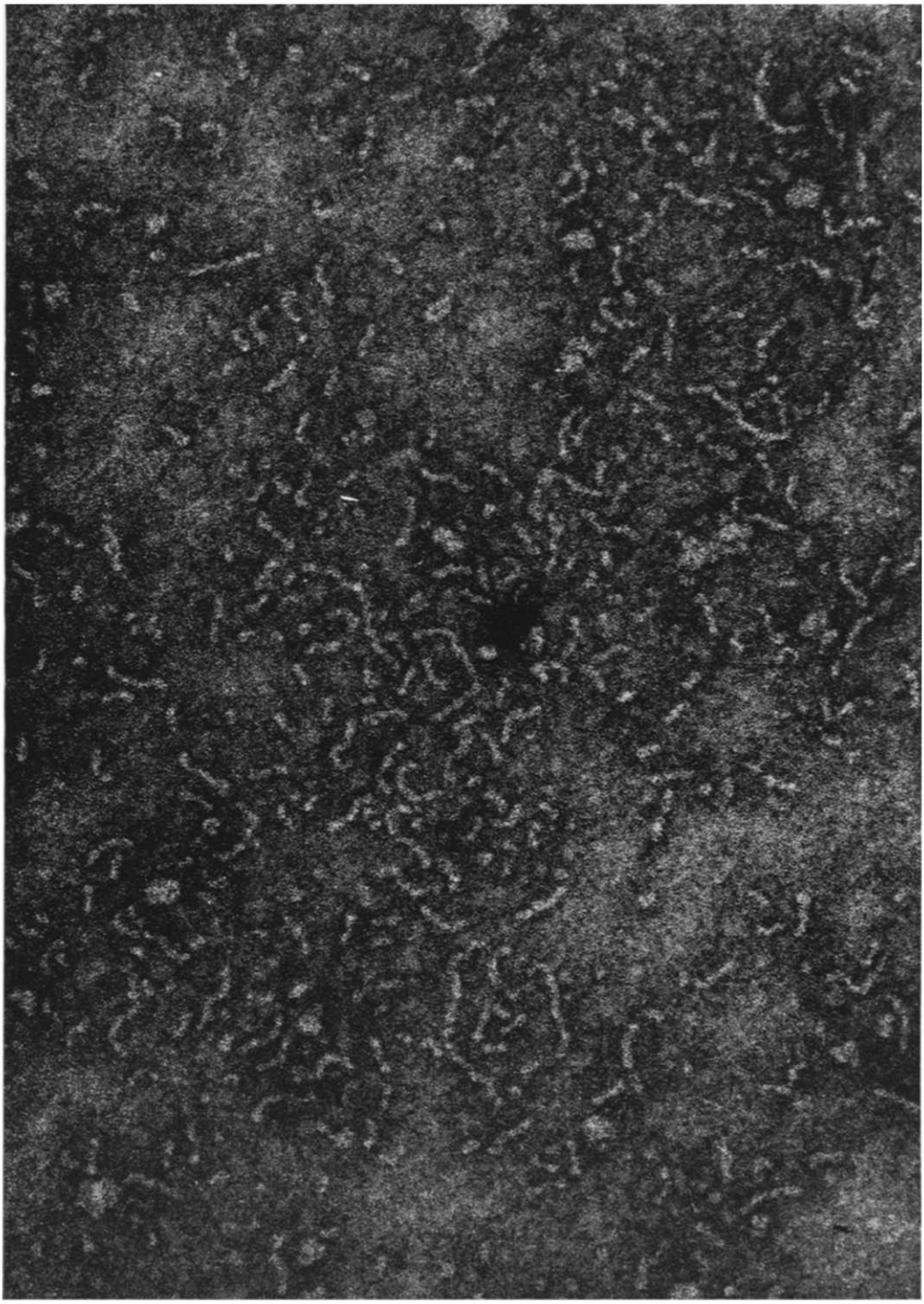

Fig. 4. Negatively stained grana Photosystem I after passing through a 120-Å filter. The photochemically active Photosystem I fraction was derived from grana membranes by digitonin digestion and passed through the filters as shown in Fig. 2. Phosphotungstic acid (2%) buffered at pH 7.2 with 0.02 M phosphate buffer was used as the negative stain. Magnification: 256000 ×.

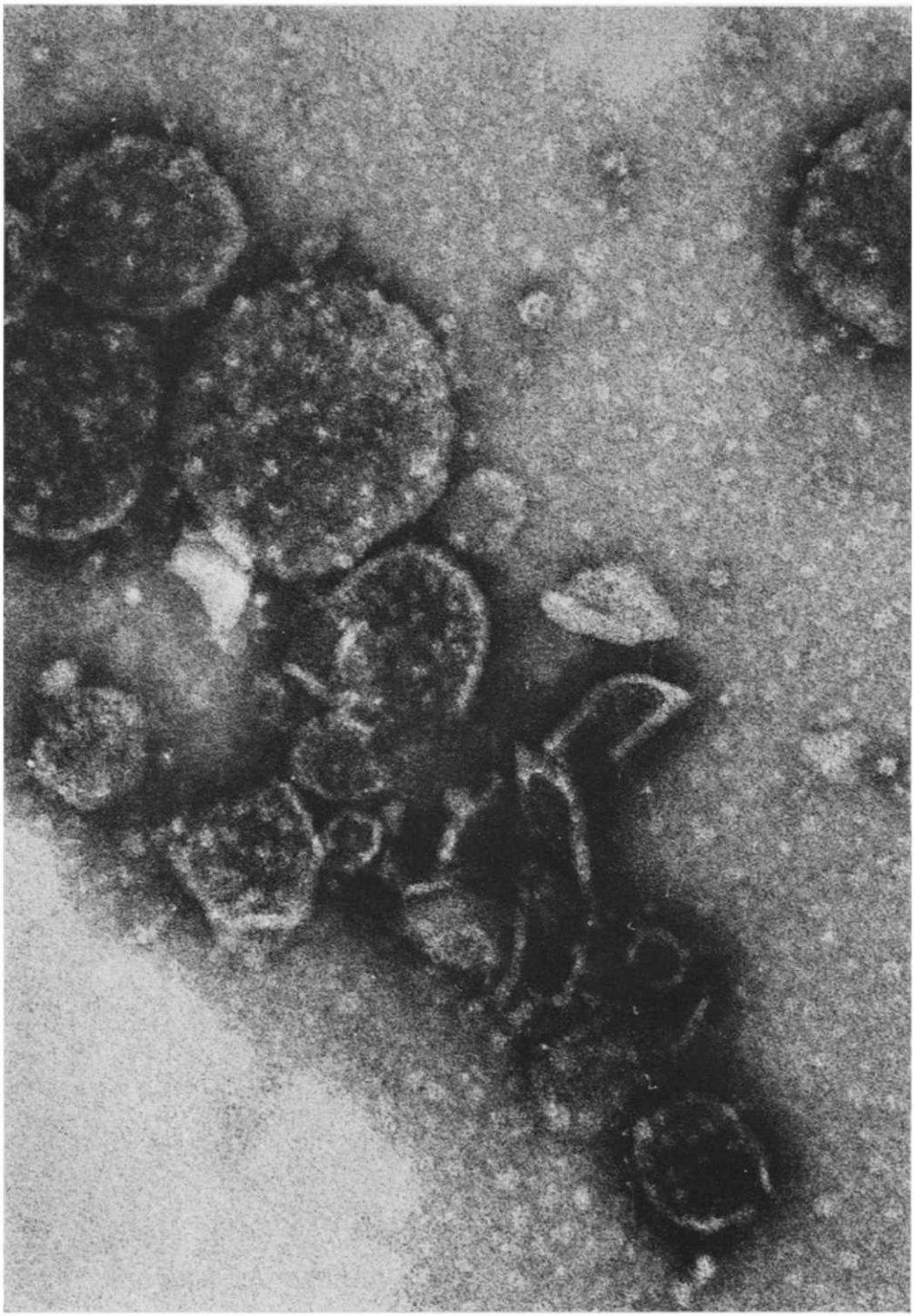

Fig. 5. Negatively stained stroma digitonin Photosystem I fraction after passage through a 120-Å filter. Conditions as for Fig. 4. Magnification: 270000 ×.

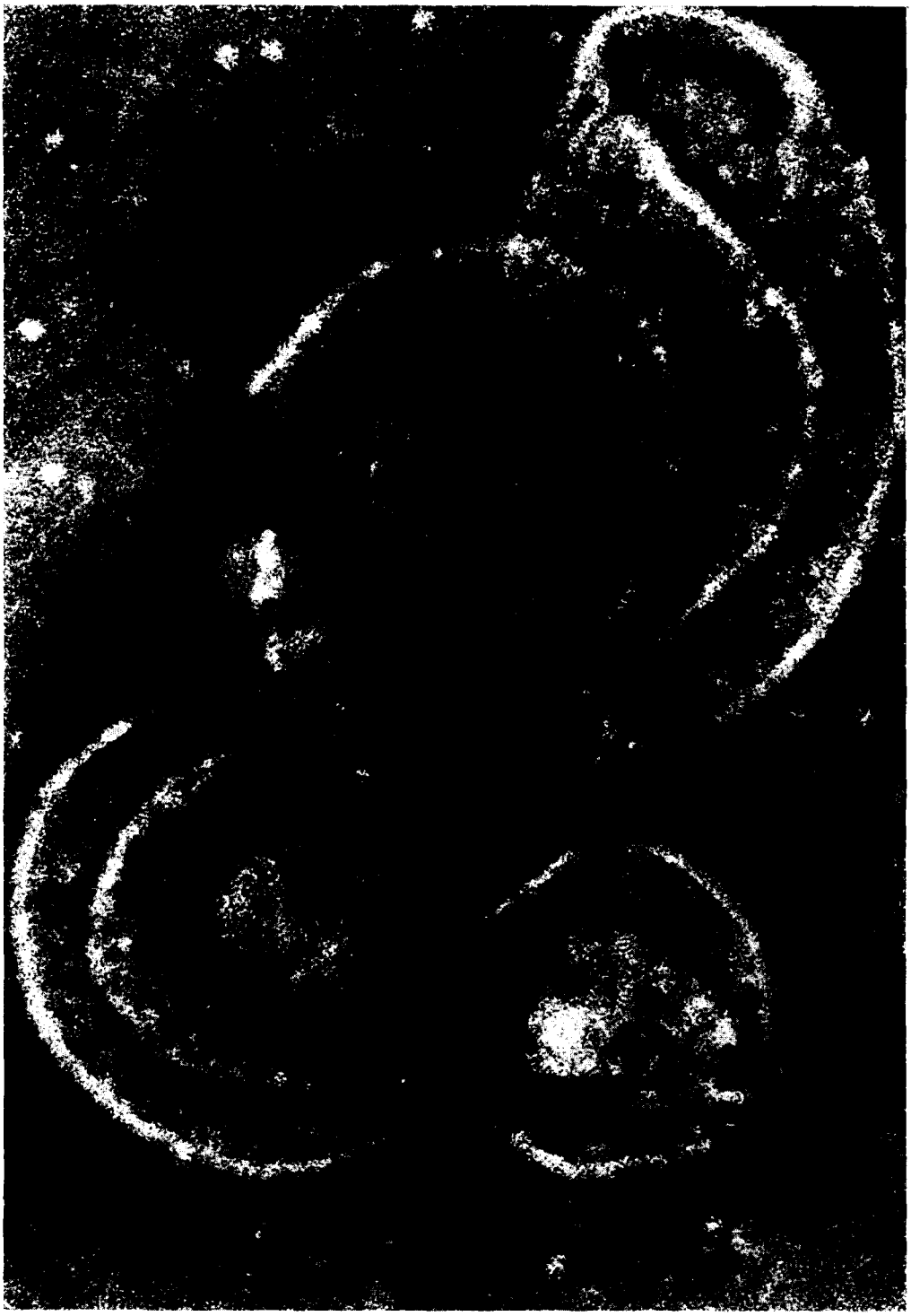

Fig. 6. Negatively stained grana Photosystem I fraction after ultrafiltration and high-salt treatment to reaggregate the membranes. Magnification: 364000 ×.

buffer (pH 7.2) and 5 mM $MgCl_2$ and centrifuged down at 144000 × *g* for 1 h, the resuspended pellet (all the green material) appeared as membranous sheets 1000–2000 Å across (Fig. 6) rather than as fiberous structures. This salt-reaggregated grana Photosystem I fraction no longer passed through even the 500-Å filter (see Table V, Line 6), a fact consistent with the membrane sheet structure shown in Fig. 6.

We have also conducted freeze–etch studies on the various membrane fractions described in this paper. In agreement with our earlier findings[14], the Photosystem I membranes from either stroma lamellae or grana Photosystem I preparations show only the small freeze–etch particles. The grana Photosystem II preparations show a predominance of large freeze–etch particles on a membranous sheet.

DISCUSSION

Grana Photosystem I and II digitonin separation

Starting with a purified grana fraction having very little stroma left, we find that digitonin gives a good separation of Photosystem II and I activities (Table I). The Photosystem I isolated from the grana makes up nearly one-half the total chlorophyll in the grana membranes, the balance being recovered in the Photosystem II fraction. The conclusion of SANE *et al.*[12] that they were unable to obtain a complete digitonin fractionation of the grana is not supported by these data for it is clear that grana Photosystem I is released in good yield by digitonin treatment. This procedure has been routinely repeated in our laboratory many times with similar chlorophyll distributions resulting. The recovery of nearly one-half the starting grana membrane chlorophyll in a Photosystem I fraction indicates that the Photosystem I has to be derived from more than just the end compartments of the grana stacks, the latter view proposed by SANE *et al.*[12]. If only the end compartments gave rise to grana Photosystem I, one would expect a much lower percentage of the grana recovered as grana Photosystem I (perhaps as low as 10 %, taking an average grana stack to have about 10 grana discs, a value from THOMAS *et al.*[33]). The earlier conclusions of ANDERSON AND BOARDMAN[4], BRIANTAIS[28], and ARNTZEN *et al.*[14] that the Photosystem I portion is on the outer part of the thylakoid membrane are supported by these present data. The point of SANE *et al.*[12] and GOODCHILD AND PARK[13] that the first action of digitonin is to remove stroma lamellae from the grana stacks is no doubt valid.

Photochemical activities of grana and stroma fractions

The photochemical activities of the grana Photosystem I and II fractions, the P700 content; and the complete recovery of the starting chlorophyllous material are inconsistent with predictions based on the ARNON *et al.*[10] hypothesis that water or diphenyl carbazide → $NADP^+$ electron transport occurs *via* two Photosystem II reactions without the involvement of Photosystem I. That hypothesis predicts that the Photosystem II fraction (our D-II), free of Photosystem I (and P700), should reduce $NADP^+$ using water or diphenyl carbazide nearly as well as it would reduce DCIP. This prediction fails as Table I shows, for the grana Photosystem II fraction (D-II) has a diphenyl carbazide → DCIP rate of 122 μmoles DCIP reduced per mg Chl per h and Table III shows that similar fractions have a diphenyl carbazide → $NADP^+$ activity of only about 15 μmoles NADPH per mg Chl per h. This latter activity was in the presence of added plastocyanin, ferredoxin, and ferredoxin reductase so those

factors were not limiting. The grana Photosystem II had an ascorbate + DCIP→ $NADP^+$ rate of 43 (Table I). The P700 content of this fraction was less than 1 P700 per 1730 chlorophylls by chemical oxidation, and less than 1 P700 per 1390 chlorophylls by the photochemical oxidation assay. The diphenyl carbazide→ $NADP^+$ and the ascorbate + DCIP→ $NADP^+$ activities present in the grana Photosystem II (D-II) fraction can be entirely accounted for by this residual P700 content. The calculations to establish this point are as follows:

Let the grana Photosystem I activity of Table I be expressed on a P700 basis *i.e.* 490 μmoles $NADP^+$ reduced per h per mg Chl × 170 mg Chl per mg P700 = $8.3 \cdot 10^4$ μmoles $NADP^+$ per mg P700 per h. This gives a calculated rate of turnover for P700 in Fraction D-I. If this same value is used for the P700 in Fraction D-II, the expected rate of $NADP^+$ reduction would be $1.2 \cdot 10^5$ μmoles $NADP^+$ per mg P700 per h × 1 mg P700 per 1730 mg Chl = 48 μmoles $NADP^+$ per mg Chl per h. This is very close to the measured rate of 43, suggesting that the grana Photosystem I activity of the "Photosystem II fraction" is due to residual P700 left in the fraction rather than being due to $NADP^+$ reduction which does not utilize P700.

Furthermore, the grana Photosystem II fraction low in P700 and low in diphenyl carbazide→ $NADP^+$ activity can be reconstituted by adding back grana Photosystem I particles *plus* exogenous lipid (Table III). The diphenyl carbazide→ $NADP^+$ activity then rises to around 65 μmoles $NADP^+$ reduced per mg Chl per h. Stroma Photosystem I, prepared identically to the grana Photosystem I was inactive in reconstituting diphenyl carbazide→ $NADP^+$ activity when added to the grana Photosystem II fraction, although both the stroma Photosystem I and grana Photosystem II were active in their respective partial reactions (Table IV). That particular grana Photosystem II used was shown to recombine with grana Photosystem I and produce more than a 2-fold increase in diphenyl carbazide→ $NADP^+$ activity. The inability of stroma Photosystem I to recombine with grana Photosystem II is evidence against the diphenyl carbazide→ $NADP^+$ electron transport being due to Photosystem II reducing a soluble redox agent present in the assay (such as plastocyanin) and that agent diffusing around until a random collision with a grana Photosystem I particle. For that explanation to hold, both grana and stroma Photosystem I preparations should suffice for activity.

In the reconstitution assay, the diphenyl carbazide→ $NADP^+$ activity was due to Photosystem II function and not just due to diphenyl carbazide donation to grana Photosystem I directly since it was completely DCMU inhibited. The necessity to mix the two types of particles while concentrated *plus* the beneficial effect of lecithin (or asolectin) is consistent with the supposition that a true recombination of the Photosystem II and I parts occurred. The lecithin requirement for optimal activity is reasonable since both freeze–etch data and the Photosystem II and I separating effect of digitonin and Triton X-100 indicate that Photosystem II and I are held together in the membrane *via* lipid–lipid interaction (at least in part). The added lecithin may restore a type of lipid interaction and lead to Photosystem II and I associating with one another. Lecithin did not stimulate diphenyl carbazide→ DCIP electron transport (a measure of Photosystem II activity only) in preparations which showed lecithin stimulation of diphenyl carbazide→ $NADP^+$ activity (Table III). That lecithin stimulated diphenyl carbazide→ $NADP^+$ in Photosystem II particles alone is possibly due to the added lipid causing a better association of what residual

Photosystem I material normally adheres to the Photosystem II membrane preparations.

The lack of stimulation in diphenyl carbazide→ $NADP^+$ activity in Photosystem II *plus* boiled grana Photosystem I particles is evidence against the stimulation being due to some additive in Photosystem I which simply stimulates further the diphenyl carbazide→ $NADP^+$ activity in the Photosystem II preparation. An active grana Photosystem I has to be present to optimally link the diphenyl carbazide oxidation through to $NADP^+$. This strongly implicates the functioning of P700 in the diphenyl carbazide (or water)→ $NADP^+$ reduction.

HUZISIGE *et al.*[27] also showed reconstitution of Photosystem II and I activity from mixing separated Photosystem II and I particles, though at considerably lower activities than reported here. BRIANTAIS[28] presented evidence for reconstitution of Triton dispersed Photosystem II and I particles by following the O_2 burst phenomenon. Our data confirm and extend that earlier work.

The action spectra data (Fig. 3) indicates that those fractions rich in P700 are also active in $NADP^+$ reduction *via* a long wavelength pigment system. It is significant that those fractions enriched in water or diphenyl carbazide oxidizing activity (the grana Photosystem II) have very low diphenyl carbazide→ $NADP^+$ activity (less than 20 μmoles $NADP^+$ per h per mg Chl, Tables III and IV) while their DCMU inhibitable diphenyl carbazide→ DCIP rates are from 100 to 400 μmoles/h per mg Chl. This again suggests that $NADP^+$ reduction capacity does not fractionate with Photosystem II and the action spectra of the other fractions indicates that no other short wavelength pigment system is recovered which could function as the Photosystem II_a postulated by ARNON *et al.*[10]. It is important to note that the Photosystem II and I fractions derived from the grana constitute 60 and 40 % of the grana material with essentially 100 % recovery of the starting materials. The good recovery of the starting materials indicates that we were not inadvertently losing a portion of the membrane, which may have been attributable to a Photosystem II_a fraction.

These several lines of evidence seem to us compelling reasons for supporting the view that water or diphenyl carbazide→ $NADP^+$ electron transport utilizes P700, or grana Photosystem I, in series with the Photosystem II photoact. This concept disagrees with recent work of RURAINSKI *et al.*[31] who found discrepancies between rates of $NADP^+$ reduction and P700 turnover, as Mg^{2+} concentration was raised from 0 to 10 mM. At high Mg^{2+} their data show low P700 and high $NADP^+$ reduction rates. In view of our present knowledge that stroma have only Photosystem I and no $NADP^+$ reduction capacity from water, the question may be raised as whether they may have been measuring only the P700 in the stroma. The P700 in the grana may not be measurable by their relaxation technique due to its possible rapid rereduction. To check such a possibility, it would be desirable to have their experiments repeated using a stroma-free grana stack preparation.

For our results to be consistent with the viewpoints of ARNON *et al.*[10] and RURAINSKI *et al.*[31] we are forced to accept at least two assumptions, the first being that our isolated grana Photosystem II fraction has lost its Photosystem II_a function during manipulation and can no longer couple with Photosystem II_b and reduce $NADP^+$, while the DTS-III preparation of ARNON *et al.*[10] maintains its Photosystem II_a activity through a much more rigorous array of detergent and physical disruption techniques. This is an unreasonable assumption as suggest by the fact that the rates

of electron transport attained by our preparations are much higher than those attained with the DTS-III preparation. The reconstitution of diphenyl carbazide→ $NADP^+$ activity by adding grana Photosystem I to the Photosystem II also is inconsistent with the $NADP^+$ reduction being due to Photosystem II_a rather than grana Photosystem I coupling with Photosystem II. The second assumption (needed to make our data consistent with that of RURAINSKI *et al.*[31]) is that the physical presence of grana Photosystem I is necessary for the electron transfer to occur between Photosystem II and $NADP^+$, but that the P700 in the grana Photosystem I is not actually involved in the electron transfer. This is an awkward assumption requiring considerable documentation to render it tenable, but we cannot rule out such a possibility since we did not measure the turnover of P700.

The fact that stroma Photosystem I did not reconstitute activity with grana Photosystem II could be due to either the stroma being deficient in a factor necessary to properly link Photosystem II and the stroma P700 reaction center (an interpretation we favor) or there may in fact be an alternative reaction center (such as ARNON's Photosystem II_a) which is absent in the stroma Photosystem I preparation but present in the grana Photosystem I or it is present in the grana Photosystem II fraction and only the grana Photosystem I fraction can, when present, elicit its function in $NADP^+$ reduction. However, such an interpretation seems rather *ad hoc* in light of our other data.

Plastocyanin in grana and stroma

Plastocyanin assays (Table II) show that both stroma lamellae and grana contain plastocyanin, at about the same concentration on a chlorophyll basis. The grana and stroma membranes assayed for about 2200 and 1500 chlorophylls respectively per plastocyanin by the bioassay we used. A similar bioassay was recently described by PLESNICAR AND BENDALL[26]. Comparing these data to the whole chloroplasts (500 chlorophylls per plastocyanin), indicates that a considerable amount of plastocyanin was lost to the medium during the manipulation. The assay of the supernatant from the 144000 × *g* centrifugation after French press treatment does show that plastocyanin is present, obviously having been extracted from the chloroplast membranes during the French press treatment and subsequent centrifugation steps. Assuming that plastocyanin was leached from grana and stroma to the same degree, we conclude that both membrane parts contain plastocyanin at about the same concentration. These data agree with recent work of HAUSKA *et al.*[25] and BASZYNSKI *et al.*[29] but disagree with results of KNAFF AND ARNON[30]. This finding makes the observation that added plastocyanin is required to sustain the best rates of electron transport in stroma and grana Photosystem I particles consistent with a functional redox role for plastocyanin in the electron transport chain of grana and stroma Photosystem I preparations.

Ultrastructure of Photosystem I

The digitonin-derived Photosystem I fractions from grana and stroma appear similar by negative staining, being irregular rods 50 Å wide by 200–500 Å long. A similar structure was shown by VERNON *et al.*[23] for Photosystem I derived by Triton X-100 fractionation. These rod structures apparently reaggregate under high salt conditions and then appear as membrane sheets (Figs. 5 and 6). Before high salt treatment the Photosystem I particles pass through 100–120-Å but not 50-Å filters. These sizes

are the upper limit of the pores, many of them being considerably smaller, which may account for the 66–85 % retention of the digitonin solubilized Photosystem I shown in Table V.

The capacity or ability of the Photosystem I particles to reaggregate as evidenced by these structural studies suggest a mechanism by which the reconstitution of diphenyl carbazide→ $NADP^+$ electron flow can be attained. According to our membrane model[14] the grana Photosystem I membrane subunits are localized on the outer half of the binary grana membrane in a position such that they can interact with Photosystem II to carry out complete non-cyclic electron flow. When these subunits are solubilized away from the membrane by detergent action, this linkage between the photosystems is, of course, broken. Reconstitution of non-cyclic electron flow (Table III) by adding together the Photosystem II membrane fragments and grana Photosystem I solubilized particles (in the presence of additional exogenous lipid) implies that the solubilized subunits can, under appropriate conditions, once again assume their normal position and reestablish a binary membrane. An interesting question, not answered by this study concerns the state of the grana Photosystem I particles when recombination takes place, *i.e.*, do the grana Photosystem I particles have to be in the dissociated state depicted by Fig. 4 or in the reaggregated state shown by Fig. 6? The fact that high salt (0.1 M KCl) which leads to formations of grana Photosystem I membrane sheets did not interfere with the reconstituted electron transport activity (G. A. PETERS, R. A. DILLEY AND E. R. SHAW, unpublished observations), makes it reasonable that reconstitution does take place with grana Photosystem I in the sheet form. This does not exclude reconstitution also taking place when the grana Photosystem I is in the dissociated form.

Membrane models

The data of this report are consistent with the general features of the membrane model we proposed earlier[14]. The model refers now to grana membranes with regard to the locus of photochemical activity, since it is now known that stroma lamellae have little or no Photosystem II. The ease of solubilization of grana Photosystem I by detergents[4,28] and the recovery of nearly 50 % of the grana chlorophyll in a reasonably pure Photosystem I fraction are strong points in favor of the binary nature of the membrane with Photosystem I on the external "half" and with all of the grana thylakoids sharing a common structure. As pointed out above, the Photosystem I cannot be localized only on the outermost grana thylakoid and still constitute 50 % of the grana mass.

The particulate nature of the grana Photosystem I as derived from digitonin action (Fig. 4) indicates that the detergent probably does not "split" the membrane into two sheets. This term was an unfortunate choice and should not be used to describe what apparently is a solubilization of particles held in a two-dimensional sheet by forces which can be disrupted easily by detergent action.

As pointed out by PARK AND SANE[32] the ARNTZEN, DILLEY, CRANE model shows a thick matrix into which grana Photosystem I and II protrude. The existence of so large a matrix region has never been clearly shown. It is presently an open question as to the molecular arrangement between Photosystem I and II within the grana membrane, but we feel that the binary nature and the location of the Photosystem I and II portions as depicted by that model are correct.

While this manuscript was in the reviewing process, new experiments were carried out using a water-soluble diazo compound as a label for external membrane portions. The results confirm that grana Photosystem I is much more exposed to the suspending media than in Photosystem II, but that both fractions are labeled to a similar extent after digitonin separation[35].

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