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PHOTOCHEMICAL PROPERTIES OF A PHOTOSYSTEM II SUBCHLOROPLAST FRAGMENT

N.K. BOARDMAN

C.S.I.R.O., Division of Plant Industry, Canberra City 2601 (Australia)

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SUMMARY

1. The Photosystem II fraction (D-10) obtained by incubation of spinach chloroplasts with digitonin was further purified by incubation with Triton X-100. The resulting Photosystem II subchloroplast fragment (DT-10) contained 1 mole of cytochrome *b*-559 per 170 moles of chlorophyll. It lacked cytochrome *f* and cytochrome b_6 and its content of P700 was low.

2. The DT-10 fragment showed only traces of photochemical activity with water as electron donor, but it was active in a Photosystem II reaction with 2,6-dichlorophenolindophenol as electron acceptor and diphenyl carbazide as donor. Photoreduction of $NADP^+$ with diphenyl carbazide as donor was negligible. There was some photoreduction of $NADP^+$ with ascorbate *plus* 2,6 dichlorophenolindophenol as donor but this activity could be accounted for by contamination with Photosystem I. These results are consistent with the Z-scheme of photosynthesis with Photosystems I and II operating in series for the reduction of $NADP^+$ from water. DT-10 subchloroplast fragments showed a light-induced rise in fluorescence yield at 20 °C in the presence of diphenyl carbazide. A light-induced fluorescence increase also was observed at 77 °K.

3. During the preparation of the DT-10 fragment, the high potential form of cytochrome *b*-559 was largely converted to a form of lower potential and C-550 was converted to the reduced state. A photoreduction of C-550 was observed at liquid-nitrogen temperature, provided the C-550 was oxidised with ferricyanide prior to cooling. Some photooxidation of cytochrome *b*-559 was obtained at 77 °K if the preparation was reduced prior to cooling, but the degree of photooxidation was variable with different preparations. C-550 does not appear to be identical with the primary fluorescence quencher, Q.

4. Photosystem I subchloroplast fragments (D-144) released by the action of digitonin were compared with Photosystem I fragments (DT-144) released from D-10 fragments by Triton X-100. There were no significant differences between D-144 and DT-144 fragments either in chlorophyll *a*/*b* ratio or in P700 content.

INTRODUCTION

Several procedures have now been used to separate the two photochemical systems of photosynthesis[1]. In the earlier studies of Boardman and Anderson[2,3]

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPC, diphenyl carbazide; TCIP, 2,3',6-trichlorophenolindiphenol.

chloroplasts were disrupted with digitonin and the fragments separated by differential centrifugation. The light fragments (D-144) were shown to have the photochemical properties of Photosystem I, while the heavy fragments (D-10) were enriched in Photosystem II. Fragments enriched in Photosystem I and Photosystem II respectively were isolated by Briantais[4] and by Vernon and his coworkers[5] by the use of Triton X-100 (reviewed in ref. 1).

Huzisige *et al.*[6] achieved a further purification of Photosystem II by treating the D-10 fragments with Triton X-100, followed by sonication and density gradient centrifugation. The Photosystem II fragments evolved oxygen in the Hill reaction with 2,6-dichlorophenolindophenol (DCIP) as acceptor, but they were inactive for the photoreduction of $NADP^+$ either with water or ascorbate–DCIP as donors. However, Arnon *et al.*[7] reported that Photosystem II fragments, prepared by a modification of the method of Huzisige *et al.*[6] were capable of photoreducing $NADP^+$ with water as electron donor. The fragments lacked cytochromes *f* and b_6 and it was concluded that they were devoid of Photosystem I. This result supports the hypothesis of Arnon and coworkers[7,8] that noncyclic electron flow from water to $NADP^+$ is driven by Photosystem II, and that Photosystem I is concerned with cyclic electron flow and cyclic phosphorylation.

It has been our experience that the method of Huzisige *et al.*[6] and the modification of Arnon *et al.*[7] give very low yields of Photosystem II fragments. This raises the question as to whether the Photosystem II fragments prepared by this method are representative of the bulk of the Photosystem II in the chloroplast.

In the present work, we have devised a rapid procedure for preparing in good yield Photosystem II fragments from the D-10 fragments. The composition and photochemical activities of our Photosystem II fragments do not support the Arnon scheme, but they are consistent with the Z-scheme with Photosystem I and Photosystem II operating in series[9].

Physical methods also have been employed to achieve a selective fragmentation of chloroplasts[1,10]. It was shown that sonication[11] or passage of chloroplasts through a French pressure cell[12] separates stroma lamellae from grana. From these studies it was concluded that stroma lamellae contain Photosystem I, while grana contain both Photosystem I and Photosystem II. On the basis of the P700 contents of stroma and grana lamellae, Sane *et al.*[12] suggested that the Photosystem I of stroma has a smaller photosynthetic unit than grana Photosystem I.

In the present studies, we have compared the Photosystem I fragments prepared by digitonin incubation of chloroplasts[3] with the Photosystem I fragments which are released from the D-10 fraction during the preparation of Photosystem II fragments.

MATERIALS AND METHODS

Preparation of fragments

Spinach chloroplasts were incubated with 0.5 % digitonin and the fragments separated by differential centrifugation as described previously[3]. To prepare Photosystem II fragments, the 10000 × *g* fraction (D-10) was resuspended in 0.05 M phosphate buffer (pH 7.2) and 0.01 M KCl, and incubated with Triton X-100 for 30 min at 0 °C. The chlorophyll concentration was 0.10 mg/ml and the optimal concen-

tration of Triton X-100 was 0.18 % After incubation with Triton X-100, the mixture was separated by differential centrifugation at the following speeds: 10000 × *g* for 30 min, 50000 × *g* for 30 min, 144000 × *g* for 1 h. In some experiments a further centrifugation of 144000 × *g* for 17 h was added to improve the yield of Photosystem I fragments. The pellets from the respective centrifugations were resuspended in 0.05 M phosphate buffer (pH 7.2). The fractions are designated by the prefix DT; for example, the fragments which sediment at 10000 × *g* are designated DT-10, those which sediment at 50000 × *g* by DT-50.

Chlorophyll *a* and chlorophyll *b* were determined by the method of Arnon[13].

Photochemical assays

For 2,3',6-trichlorophenolindophenol (TCIP) reduction the reaction mixture contained, in 3 ml, fragments containing 10–15 μg chlorophyll and (in μmoles) Tris–HCl buffer (pH 7.8), 40; NaCl, 70; TCIP, 0.06. The decrease in absorbance at 620 nm was measured after illumination for 45 s with saturating white light.

Photosystem II activity was also assayed by the method of Vernon and Shaw[14] with diphenyl carbazide (DPC) as electron donor and DCIP as acceptor. The reaction mixture contained in 3 ml, fragments containing 10–15 μg chlorophyll and (in μmoles) phosphate buffer (pH 6.8), 90; sucrose, 750; DPC, 1.5.

For $NADP^+$ reduction, the reaction mixture contained in 0.6 ml, fragments containing 3–5 μg chlorophyll and (in μmoles) Tris–HCl buffer (pH 8.0), 8; NaCl, 14; $MgCl_2$, 2; $NADP^+$, 0.12; and saturating amounts of ferredoxin, plastocyanin and NADP reductase. Where indicated, DPC (0.3 μmole) or sodium ascorbate (2 μmoles) and DCIP (0.06 μmole) were added as electron donors. In some experiments, 0.035 % Triton X-100 was added[14]. Illumination was for 2 min with saturating white light and the amount of $NADP^+$ reduced was calculated from the absorbance increase at 340 nm. When DPC was used as donor, the absorbance increase was multiplied by 0.66 in order to correct for the oxidation of DPC (ref. 15).

P700 was determined, as described previously[16] from the light-induced absorbance change at 698 nm, determined in an Aminco-Chance dual-wavelength spectrophotometer, and from the ferricyanide oxidised *minus* ascorbate reduced difference spectrum, measured in a Cary 14R recording spectrophotometer. Cytochrome difference spectra were measured at room temperature and at 77 °K as described previously[17,18]. Absorption spectra and light-induced cytochrome changes[19] at 77 °K were measured in a Cary spectrophotometer.

Fluorescence emission and excitation spectra were made on a fluorescence spectrometer, and automatically corrected for photomultiplier and monochromator responses, and variation in output of the light source[20]. Fluorescence kinetics were measured at 683 nm[20].

RESULTS

Effect of Triton X-100 concentration in the preparation of Photosystem II fragments

Preliminary experiments with spinach chloroplasts indicated that the Hill reaction with TCIP as electron acceptor was almost completely inhibited (98 %) by incubation of the chloroplasts with 0.1 % Triton X-100 and a Triton/chlorophyll ratio of 10 (w/w). This result is in agreement with the earlier studies of Vernon and Shaw[21].

Incubation of the D-10 fragments with Triton X-100 at concentrations below 0.1 % did not produce any further enrichment in Photosystem II as compared with the digitonin fragmentation of chloroplasts. Previously[3], it was estimated that the D-10 fragments contained 70 % Photosystem II and 30 % Photosystem I. It thus seemed unlikely that we would be successful in preparing Photosystem II fragments which were free of Photosystem I but capable of photochemical oxygen evolution. Therefore, the method of Vernon and Shaw[14] was used to assay Photosystem II, with DPC as electron donor. In examining the effect of Triton X-100 concentration on the further fragmentation of the D-10 fraction, our aim was to prepare Photosystem II fragments in relatively high yield.

Table I shows a typical experiment in which D-10 fragments were incubated with 0.18 % Triton X-100. In this particular experiment the supernatant from the centrifugation at 144000 × *g* for 1 h was centrifuged for a further period of 17 h at 144000 × *g*. The resulting pellet was overlayed with a loosely packed layer of chlorophyll containing particles (the loose pellet). The supernatant was carefully removed by a syringe and the loose pellet collected as a separate fraction. The DT-10 fraction had a chlorophyll *a*/*b* ratio of 2.00 and it contained 38 % of the chlorophyll of the D-10 fraction. Over a number of experiments the chlorophyll *a*/*b* ratio of the DT-10 fraction varied from 1.8–2.0 and the yield ranged from 15–50 % of the chlorophyll of the D-10 fraction. It was generally observed that a lower chlorophyll *a*/*b* ratio was associated with a lower yield. Increasing the Triton X-100 concentration above 0.2 % substantially decreased the yield of the DT-10 fragments without a further decrease in the chlorophyll *a*/*b* ratio below 1.8.

TABLE I

FRACTIONATION OF D-10 FRAGMENTS WITH TRITON X-100

D-10 fragments were incubated with 0.18% Triton X-100 for 30 min at 0 °C and the mixture separated by differential centrifugation as described in Materials and Methods.

Fraction	*Chlorophyll distribution* (%)	*Chlorophyll a/b ratio*
D-10	(100) *	2.40
DT-10	38.5	2.00
DT-50	18.8	2.15
DT-144 (1 h)	1.7	5.29
DT-144 (17 h)	5.1	5.90
Loose pellet	9.6	3.18
Supernatant	16.7	2.59

* 43% of the chlorophyll of the chloroplasts before incubation with digitonin.

The small fragments which sedimented at 144000 × *g* (1-h and 17-h pellets) had a high chlorophyll *a*/*b* ratio indicative of Photosystem I fragments. The yield of Photosystem I fragments varied from 7–10 % of the chlorophyll of the D-10 fraction. The loose pellet and the 144000 × *g* supernatant (17 h) had considerable lower chlorophyll *a*/*b* ratios than did the DT-144 fraction.

Composition of DT-10 fragments

P700. P700 was detectable in the DT-10 fragments, but the amount was low and it was difficult to obtain reliable quantitative data. The light-induced method gave chlorophyll/P700 ratios in the range 3000–6000, whereas ratios in the range 2000–4000 were obtained by the chemical method (Table II). These measurements indicate that the P700 content of DT-10 fragments on a chlorophyll basis was 4–9 % of the P700 content of the Photosystem I fragments (D-144 or DT-144).

TABLE II

COMPOSITION OF DT-10 FRACTION

Chlorophyll *a*/*b*	1.8–2.0
Chlorophyll *a* + *b*/P700 (light induced)	3000–6000
Chlorophyll *a* + *b*/P700 (oxidised *minus* reduced)	2000–4000
Chlorophyll *a* + *b*/cytochrome *b* 559	170
Cytochromes *f* and b_6	Not detectable
Fluorescence (Φ_{735}/Φ_{total})	0.35–0.45

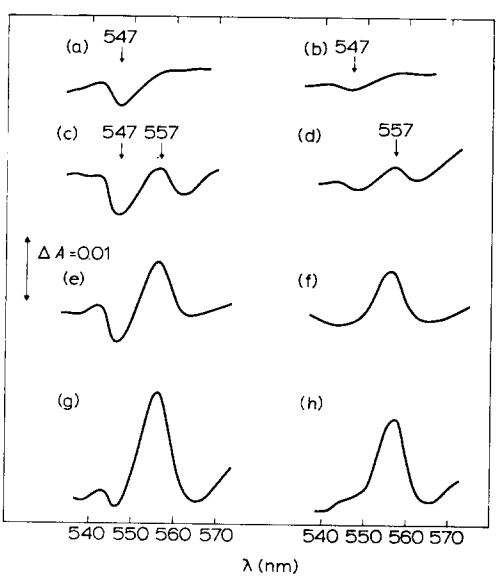


Fig. 1.Cytochrome difference spectra at 77 °K of DT-10 fragments. (a), (b), (c) and (d) untreated *minus* ferricyanide oxidised; (e) and (f), ascorbate reduced *minus* ferricyanide oxidised; (g) and (h), dithionite reduced *minus* ferricyanide oxidised. (a), (c), (e) and (g) were recorded before illumination and (b), (d), (f) and (h) after illumination at 77 °K for 3 min at 650 nm in the Cary spectrophotometer. The chlorophyll concentration was 0.28 mg/ml for (a) and (b) and 0.35 mg/ml for the other spectra. Pathlength, 2 mm. Curves (a) and (b) were recorded with a different preparation than that used for curves (c) and (d).

Cytochromes. Oxidised *minus* reduced difference spectra of DT-10 fragments at 77 °K are shown in Figs 1 and 2. Previously[18], it was shown that the spectral bands due to cytochromes *f*, b_6 and *b*-559 in chloroplasts and subchloroplast fragments are

resolved at liquid nitrogen temperature but not at room temperature. The difference spectra of DT-10 fragments show no evidence for a band at 552 nm due to cytochrome *f* or a band at 561 nm which could be attributable to cytochrome b_6. The positive band in the ascorbate reduced *minus* ferricyanide reduced spectrum (Curve e, Fig. 1) and the dithionite reduced *minus* ferricyanide reduced spectrum (Curve g, Fig. 1), and the band in the ascorbate reduced *minus* untreated and dithionite reduced *minus* untreated spectra (Curves a and b, Fig. 2) are symmetrical with a maximum at 556–557 nm. We conclude that the DT-10 fragments contain cytochrome *b*-559 but cytochromes *f* and b_6 are not detectable.

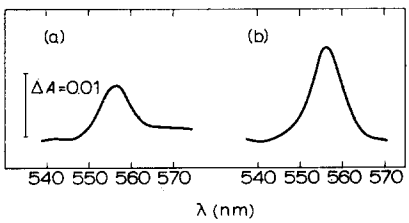


Fig. 2. (a) Ascorbate reduced *minus* untreated and (b) dithionite reduced *minus* untreated difference spectra at 77 °K of DT- 10 fragments. The chlorophyll concentration was 0.28 mg/ml and the pathlength 2 mm.

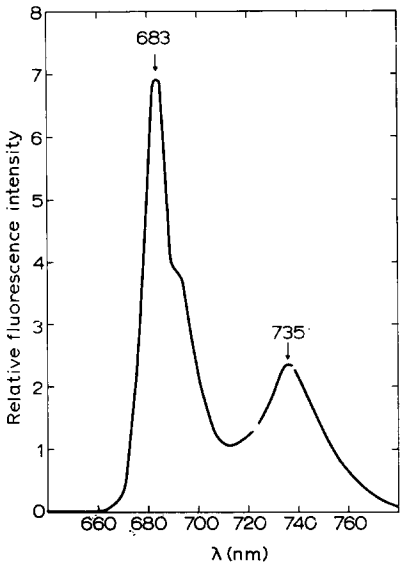
In our preparations of spinach chloroplasts, the high potential form of cytochrome *b*-559 is mainly (>90 %) in the reduced state[18]. However, in preparations of DT-10 fragments, cytochrome *b*-559 was mainly in the oxidised state, as indicated by the untreated *minus* ferricyanide oxidised difference spectra (Curves a and c, Fig. 1). In the preparation shown by Curve a, cytochrome *b*-559 was not detectable in the untreated *minus* ferricyanide oxidised spectrum, but it is present in Curve c from another preparation. The fraction of cytochrome *b*-559 in the oxidised state varied from 70–100 %. It is now established that the high potential form of cytochrome *b*-559 in chloroplasts is sensitive to a variety of treatments[22,23], including heat, solvent extraction and treatment with Triton X-100. These treatments convert the high potential form to an autoxidizable form of lower potential which is largely reducible by ascorbate but not by hydroquinone. In the preparations of DT-10 fragments, the oxidised fraction of cytochrome *b*-559 was not reduced by hydroquinone; about two-thirds was reduced by ascorbate and the remainder by dithionite.

The amount of cytochrome *b*-559 in DT-10 fragments was determined from the dithionite reduced *minus* ferricyanide oxidised difference spectrum at room temperature, using a molar extinction coefficient of $2.0 \cdot 10^4$. A chlorophyll/cytochrome *b*-559 molar ratio of 170 was obtained (Table II).

The untreated *minus* ferricyanide oxidised, ascorbate reduced *minus* ferricyanide oxidised, and dithionite reduced *minus* ferricyanide oxidised difference spectra at 77 °K show a negative band at 547 nm, due to the component, C-550, discovered by Knaff and Arnon[24], and considered to be the primary electron acceptor of Photosystem II. In isolated chloroplasts, C-550 is in the oxidised state; it is reduced by dithionite, but not by ascorbate[19,24–26]. However, C-550 in DT-10 fragments appears to be in the reduced state, as indicated by the presence of the negative band at

547 nm in the untreated *minus* ferricyanide spectra (Fig. 1), and the absence of a negative band at 547 nm in the dithionite reduced *minus* untreated spectrum (Fig. 2).

Fluorescence spectra. On illumination of DT-10 fragments at 77 °K, the fluorescence emission at 683 nm rises in an exponential manner from an initial level to a steady-state level which is 3-fold higher. The steady-state fluorescence emission spectrum at 77 °K is shown in Fig. 3. The 735 nm band accounts for about 40 % of the total fluorescence emission, compared with 60 % for D-10 fragments and 75 % for chloroplasts[27]. The fluorescence excitation spectrum of DT–10 fragments (Fig. 4) shows its main peak at 674 nm and a minor band at 648 nm, due to chlorophyll *b*.



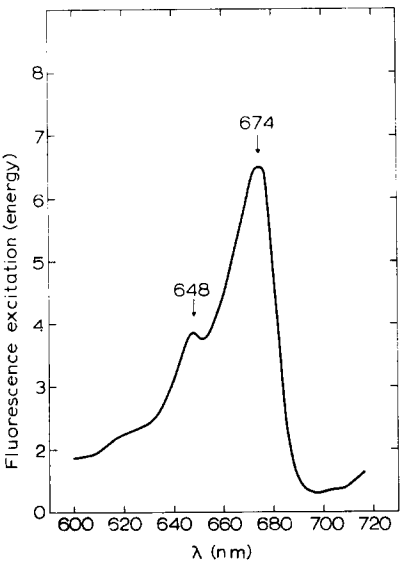


Fig. 3. Steady-state fluorescence emission spectrum of DT-10 fragments at 77 °K. Excitation wavelength, 436 nm. Concentration of fragments, 0.1 $A_{436\ nm}$ unit.

Fig. 4. Steady-state fluorescence excitation spectrum of DT-10 fragments at 77 °K. Emission wavelength, 735 nm. Concentration of fragments, 0.1 $A_{436\ nm}$ unit.

TABLE III

PHOTOCHEMICAL ACTIVITIES OF DT-10 FRAGMENTS

Photochemical activities were assayed under saturating light as described in Materials and Methods. The concentration of DCMU was $1 \cdot 10^{-5}$ M.

Reaction	*Activity (μmoles/mg chlorophyll per h)*		
	−DCMU	*+DCMU*	*DCMU-sensitive*
$H_2O \rightarrow$ TCIP	6.9	0.3	6.6
$H_2O \rightarrow NADP^+$	6.5	1.5	5.0
DPC → $DCIP^+$	103.3	39.9	63.4
DPC → $NADP^+$	5.1	3.6	1.5
DPC → $NADP^+$ (0.035% Triton X-100)	15.1	9.0	6.1
Ascorbate–DCIP → $NADP^+$	8.0	7.3	0.7
Ascorbate–DCIP → $NADP^+$ (0.035% Triton X-100)	23.5	22.2	1.3

The main peak is shifted 5 nm to a shorter wavelength compared with the excitation maximum for D-144, Photosystem I fragments[27]. Its position agrees with the maximum in the action spectrum for Photosystem II, as determined recently by Fork[28] for the photooxidation of cytochrome *b*-559 in fragments enriched in Photosystem II. The absorption spectrum of DT-10 fragments at 77 °K (Fig. 5) resembles the fluorescence excitation spectrum.

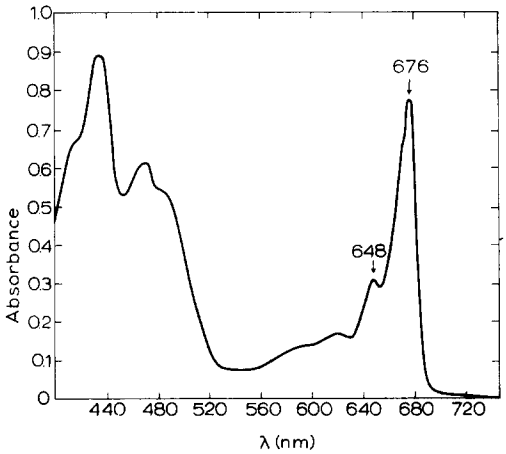


Fig. 5. Absorption spectrum of DT-10 fragments at 77 °K. Chlorophyll concentration, 37.2 μg/ml. Pathlength, 2 mm.

Photochemical activities of DT-10 fragments

Electron transport reactions at 20 °C. A representative experiment is shown in Table III. The rate of TCIP reduction with water as electron donor was low amounting to only about 2 % of the rates observed with chloroplasts. A similar low rate of $NADP^+$ reduction was observed. However, DT-10 fragments showed good Photosystem II activity with DPC as donor and DCIP as acceptor. DCIP reduction was inhibited about two-thirds by 3-(3,4-dichlorophenyl)-1,1 dimethylurea (DCMU). The rate of DCIP reduction by DT-10 fragments was comparable to that observed by chloroplasts in our experiments.

TABLE IV

PHOTOCHEMICAL ACTIVITIES OF DT-144 FRAGMENTS

DT-144 fragments were prepared by sedimentation for 1 h at 144000 × *g* as described in Materials and Methods.

Reaction	*Activity (μmoles/mg chlorophyll per h)*	
	−DCMU	*+DCMU*
DPC → DCIP	17.4	15.0
DPC → DCIP (0.035% Triton X-100)	34.1	36.0
DPC → $NADP^+$	4.2	3.7
DPC → $NADP^+$ (0.035% Triton X-100)	30.2	33.5
Ascorbate + DCIP → $NADP^+$ (0.035% Triton X-100)	610	580

The Photosystem II fragments showed relatively low rates of $NADP^+$ photoreduction either with water or DPC as electron donor. There was a stimulation of $NADP^+$ reduction by 0.035 % Triton X-100, but two-thirds of the activity was insensitive to inhibition by DCMU. The DT-10 fragments showed some Photosystem I activity as measured by the photoreduction of $NADP^+$ with ascorbate + DCIP as electron donor, but the rate of $NADP^+$ reduction was low (approx. 4 %) compared with that of the Photosystem I, DT-144 fragments (Table IV). DT-144 fragments showed some photoreduction of DCIP and $NADP^+$ with DPC as donor, but these reactions were insensitive to DCMU.

TABLE V

FLUORESCENCE PROPERTIES OF DT-10 FRACTION

Fluorescence was excited at 436 nm and the time course of fluorescence emission measured at 683 nm. F_∞ was the steady-state level. The fluorescence emission at 683 nm was also measured in the presence of $5 \cdot 10^{-5}$ M ferricyanide ($F_{oxidised}$) or dithionite ($F_{reduced}$). Fluorescence amplitudes were measured with the electrical gain inversely proportional to light intensity. Temperature, 20 °C. The concentration of DT-10 fragments was 0.20 $A_{436\ nm}$ unit. DPC was added to a final concentration of $0.5 \cdot 10^{-3}$ M.

Excitation Light intensity ($erg \cdot cm^{-2} \cdot s^{-1}$)	*Diphenyl carbazide*	$F_{oxidised}$	F_∞	$F_{reduced}$	$\frac{F_{reduced}}{F_{oxidised}}$	$\frac{F_{reduced}}{F_\infty}$
90	−	52	126	258	5.0	2.0
270	−	54	118	256	4.7	2.3
900	−	60	124	250	4.2	2.0
90	+		200	250		1.3
270	+		200	224		1.1
900	+		210	230		1.1

Fluorescence properties at 20 °C. The light-induced increase in the fluorescence emission of isolated chloroplasts is an indication of electron flow in Photosystem II (ref. 29). The fluorescence kinetics of DT-10 fragments are summarized in Table V. It was difficult to determine the initial level of fluorescence (F_0) with our present apparatus because of the rapid rise in the fluorescence intensity of DT-10 fragments on illumination, as compared with chloroplasts. Table V reports values of $F_{oxidised}$ instead of F_0. In the case of chloroplasts $F_{oxidised}$ is equal to F_0 (ref. 20).

The $F_{reduced}/F_{oxidised}$ ratio of DT-10 fragments is similar to that of chloroplasts, but the steady state level of fluorescence is considerably below the $F_{reduced}$ level even at the highest light intensity used in our experiments. This result which is consistent with the low rates of electron flow from water for DT-10 fragments suggests that the primary fluorescence quencher of Photosystem II is only partly reduced in the light. On addition of DPC, F_∞ approached the $F_{reduced}$ level particularly at higher light intensities.

Light-induced absorbance changes at liquid-nitrogen temperature. Knaff and Arnon[30], reported that cytochrome *b*-559 is photooxidised at 77 °K by Photosystem II. They also observed a light-induced decrease at 548 nm at 77 °K which was attributed to the photoreduction of a new component, C-550, by Photosystem II[24]. These observations have been confirmed in several laboratories[18,25,26,31], and it was further

suggested that cytochrome *b*-559 can act as an electron donor for C-550, and that C-550 is identical to the fluorescence quencher, Q[19,26,32].

The experiment reported in Fig. 1 indicates light-induced absorbance changes at 557 nm (due to cytochrome *b*-559) and at 547 nm (due to C-550) in DT-10 fragments which were illuminated at 77 °K by 650 nm light. The following procedure was used to determine light-induced changes at liquid-nitrogen temperature. The sample was frozen to 77 °K in a dim green light and a reduced *minus* oxidised difference spectrum recorded. Both reference and sample cuvettes were then illuminated at 650 nm for 3 min (*cf.* ref. 19) and the spectrum again recorded. A comparison of the ascorbate reduced *minus* ferricyanide oxidised difference spectra (Curves e and f, Fig. 1) shows a small decrease at 557 nm on illumination and a more marked increase at 547 nm. Curves g and h are dithionite reduced *minus* ferricyanide oxidised difference spectra recorded before and after illumination. The light-induced decrease in absorbance at 557 nm is of greater magnitude than indicated by Curves e and f, suggesting that cytochrome *b*-559 of low potential (dithionite reducible) is undergoing photooxidation. The extent of the photooxidation of cytochrome *b*-559 was variable from preparation to preparation, and in some preparations photooxidation of cytochrome *b*-559 at 77 °K could not be detected.

The increase in absorbance at 547 nm is attributed to the photoreduction of C-550, the reduction occurring in the reference cuvette, where the sample had been oxidised with ferricyanide prior to cooling. This conclusion was confirmed from light *minus* dark difference spectra. If sample and reference samples were oxidised with ferricyanide prior to cooling, the light *minus* dark difference spectrum showed a negative band at 547 nm, due to the bleaching of C-550. But if the sample and reference samples were untreated or reduced with ascorbate, then no bleaching of C-550 was observed at 77 °K. This latter result is in contrast to experiments with chloroplasts, and confirms the conclusion that C-550 is in the reduced state in DT-10 fragments. Photoreduction of C-550 was observed in all preparations of DT-10 fragments, irrespective of whether photooxidation of cytochrome *b*-559 was detectable.

TABLE VI

CHLOROPHYLL/P700 RATIOS OF D-144 AND DT-144 FRACTIONS

D-144 and DT-144 fractions were prepared as described in Materials and Methods. P700 was determined from the light-induced decrease in absorbance at 698 nm(photochemical method) or from a ferricyanide oxidised *minus* ascorbate reduced difference spectrum (chemical method). Average values are given in parentheses.

Fraction	*Chlorophyll/P700*							
	Photochemical method				*Chemical method*			
D-144	240,	230,	220	(230)	218,	170,	180	(190)
DT-144 (1 h pellet)	270,	280,	222	(257)	164,	171	(168)	
DT-144 (17 h pellet)	260				150			
Chloroplasts	460				425			

P-700 content of D-144 and DT-144 fractions

A comparison of the chlorophyll/P700 ratio of Photosystem I fragments released by digitonin treatment of chloroplasts with that of the DT-144 fraction is shown in

Table VI. There appear to be no significant differences between the corresponding ratios; the photochemical method giving slightly higher chlorophyll/P700 ratios for the DT-144 fraction and the chemical method somewhat lower values. It has been our experience, however, that the chemical method gives significantly lower chlorophyll/P-700 ratios for Photosystem I fragments than the photochemical method.

DISCUSSION

Sane *et al.*[12] demonstrated that passage of chloroplasts through the French Press separates stroma lamellae from grana. They proposed that Photosystem I fragments prepared in this way[33] originate from stroma lamellae and possibly end grana membranes, whereas the fraction enriched in Photosystem II originates from the grana regions. Similar conclusions were reached by Jacobi and Lehmann[11] for the origin of Photosystem I released by short term sonication of chloroplasts. Goodchild and Park[34] suggested that the Photosystem I fraction obtained by digitonin incubation also originates from stroma lamellae. We agree with the conclusion that the initial action of digitonin is to separate stroma lamellae from grana. We consider, however, that in the incubations with digitonin as used originally by Boardman and Anderson[2,3] some Photosytem I was released from grana[35]. It has been our experience that the yield of Photosystem I is considerably higher with digitonin incubation than by passage of chloroplasts through the French Press, particularly when the yields and chlorophyll composition of the 50 000 $\times$ *g* fractions are taken into consideration[35]. The recent studies of Arntzen *et al.*[15] demonstrate conclusively that Photosystem I can be released from grana by the action of digitonin.

On the basis of the P700 contents of chloroplasts and Photosystem I fragments released from stroma lamellae, Sane *et al.*[12] suggested that Photosystem I in the stroma has a smaller photosynthetic unit size than Photosystem I in the grana. In the present work, no significant difference was observed between the Photosystem I released by digitonin and the Photosystem I released by action of Triton X-100 on the D-10 fraction. It is apparent, however, that the chemical method of determining P700 gives relatively higher contents of P700 than the photochemical method, particularly for the Photosystem I fragments. The chlorophyll *a*/*b* ratios of the Photosystem I fragments released by digitonin and Triton X-100, respectively, did not differ significantly.

We estimate that the DT-10 fragments contain about 7–15 % Photosystem I depending on the preparation. This estimate is obtained from a consideraton of the fraction of fluorescence emitted at the 735 nm band at 77 °K and from the chlorophyll *a*/*b* ratio. The P700 content indicates a Photosystem I contamination of 4–9 %, but as mentioned earlier, it is difficult to estimate low levels of P700. It is not known whether this more tightly bound Photosystem I is identical to the Photosystem I released by digitonin and Triton X-100. In their recent studies, Arntzen *et al.*[15] obtained an excellent fractionation of the grana fraction by digitonin treatment. The grana Photosystem I fragments had a somewhat lower content of P700 and a lower chlorophyll *a*/*b* ratio than the stroma Photosystem I fragments.

In contrast with the Photosystem II fragments prepared by Husizige *et al.*[6] and Arnon *et al.*[7] the DT-10 fraction did not evolve oxygen in the Hill reaction, but it showed good Photosystem II activity with DPC as electron donor. The Photosystem I activity of the DT-10 fraction was low compared with the activity of the DT-144

fraction, and it can be accounted for by the contamination of DT-10 fragments by some residual Photosystem I. Cytochromes *f* and b_6 were not detectable in DT-10 fragments but the content of P700 appeared sufficient to account for the Photosystem I activity. The Photosystem II fragments prepared by Arnon *et al.*[7] had little or no P700, but they showed considerable Hill activity with $NADP^+$ as oxidant. This result was used as evidence to support a scheme[36,7] in which $NADP^+$ reduction from water was driven by Photosystem II, which consisted, however, of two short-wavelength light relations. Recently, Malkin[37] reported a rapid method for obtaining a Photosystem II fraction by fragmentation of chloroplasts with Triton X-100. The fraction contained no detectable cytochrome *f*, cytochrome b_6 or P700. It showed comparable Photosystem II activity, with DPC as donor and DCIP as acceptor, to that of DT-10 fragments reported in this paper. The fraction was unable to photoreduce $NADP^+$ from water but considerable activity (110 μmoles $NADP^+$ reduced/mg chlorophyll per h) was observed if ascorbate *plus* plastocyanin was used as electron donor. Because P700 was not detectable in the fraction, Malkin[37] concluded that $NADP^+$ photoreduction was driven by Photosystem II. However, in view of the very high rates which have been observed for $NADP^+$ photoreduction (with ascorbate + DCIP as donor) by Photosystem I fragments prepared by Triton X-100 (2000 μmoles $NADP^+$ reduced/mg chlorophyll per h[5]), it seems possible that the rates observed by Malkin[37] for the Photosystem II fraction may still be explained by contamination with some Photosystem I.

In their studies on the fractionation of grana by digitonin, Arntzen *et al.*[15] concluded that the $NADP^+$-reduction activity of their Photosystem II fragments could be accounted for by residual P700 and Photosystem I. They also demonstrated that the Photosystem II fraction which was low in DPC→$NADP^+$ activity could be reconstituted with grana Photosystem I particles to give a reasonable $NADP^+$-photoreduction activity.

It has been suggested from studies with chloroplasts that the component C-550 is identical to Q, the primary quencher of fluorescence in Photosystem II (ref. 32). In isolated chloroplasts, both C-550 and Q are apparently in the oxidised state and neither is reduced by ascorbate. Erixon and Butler [32] estimated a midpoint potential of −50 mV for the reduction either of C-550 or Q. However, in broken chloroplasts the midpoint potential was about +25 mV.

The present studies suggest that C-550 is not identical with Q, since C-550 is in the reduced state in DT-10 fragments whereas Q appears to be oxidised. Photoreduction of Q occurs at room temperature if DPC is present as an electron donor to photosystem II. The light-reduced rise in fluorescence of DT-10 fragments at liquid nitrogen temperature, suggests that Q is photoreducible at 77 °K. C-550 is photoreduced at 77 °K provided the DT-10 fragments are treated with ferricyanide prior to freezing. It is apparent that C-550 and Q, although not identical are in close proximity to the reaction centre of Photosystem II, in view of their photoreduction at 77 °K. Perhaps C-550 is either on a side path on the reducing side of Photosystem II or on an alternative electron transport pathway between Photosystems II and I.

Studies with chloroplasts[19,26,32] indicate that cytochrome *b*-559 can act as an electron donor for the photoreduction of C-550 at 77 °K. However, cytochrome *b*-559 is not an obligate donor since C-550 photoreduction occurred even when cytochrome *b*-559 was oxidised with ferricyanide prior to freezing[24]. On the other hand, the amount of cytochrome *b*-559 which was photooxidised at 77 °K appeared to be limited by the

pool of C-550 (ref. 32) suggesting that C-550 is essential for the oxidation of cytochrome *b*-559. In all preparations of DT-10 fragments so far examined C-550 was in the reduced state and, therefore, it does not seem that C-550 was the electron acceptor for the photooxidation of cytochrome *b*-559 which was observed in some of the preparations of DT-10 fragments at 77 °K. Floyd *et al.*[31] observed a correspondence between the kinetics of photooxidation of cytochrome *b*-559 at 77 °K and the decay of the light-induced absorption of cytochrome *b*-559 at 77 °K and the decay of the light-induced absorption change at 680 nm. They suggested that P680, the reaction centre chlorophyll of Photosystem II is the primary oxidant of cytochrome *b*-559 at low temperature.

In a recent paper, Ke *et al.*[38] reported a room-temperature photoreduction of cytochrome *b*-559 in a Photosystem II reaction centre particle which was obtained from spinach chloroplasts by Triton treatment. The particle, which had a chlorophyll *a*/*b* ratio of 8 and contained 1 mole of cytochrome *b*-559 per 50 moles of chlorophyll was unable to photooxidise cytochrome *b*-559 at liquid-nitrogen temperature.

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