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ISOLATION AND CHARACTERIZATION OF PHOTOSYSTEM I AND II MEMBRANE PARTICLES FROM THE BLUE-GREEN ALGA, *SYNECHOCOCCUS CEDRORUM*

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Summary

Fractions enriched in either Photosystem I or Photosystem II activity have been isolated from the blue-green alga, *Synechococcus cedrorum* after digitonin treatment. Sedimentation of this homogenate on a 10—30% sucrose gradient yielded three green bands: the upper band was enriched in Photosystem II, the lowest band was enriched in Photosystem I, while the middle band contained both activities. Large quantities of both particles were isolated by zonal centrifugation, and the material was then further purified by chromatography on DEAE-cellulose.

The resulting Photosystem II particles carried out light-induced electron transport from semicarbizide to ferricyanide of over 2000 μmol/mg Chlorophyll per h (which was sensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea), and was nearly devoid of Photosystem I activity. This particle contains β-carotene, very little phycocyanin, has a chlorophyll absorption maximum at 675 nm, and a liquid N_2 fluorescence maximum at 685 nm. The purest Photosystem II particles have a chlorophyll to cytochrome *b*-559 ratio of 50 : 1. The Photosystem I particle is highly enriched in *P*-700, with a chlorophyll to *P*-700 ratio of 40 : 1. The physical structure of the two Photosystem particles has also been studied by gel electrophoresis and electron microscopy. These results indicate that the size and protein composition of the two particles are distinctly different.

Introduction

The blue-green algae are procaryotic organisms which perform an aerobic photosynthesis similar in many respects to that of green plants [1,2]. The

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Abbreviations: PS, Photosystem; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPIP, 2,6-dichlorophenolindophenol; Chl, chlorophyll; SDS, sodium dodecyl sulfate.

photosynthetic apparatus of blue-green algae contains chlorophyll *a*, β-carotene, cytochromes and other photosynthetic cofactors that resemble those in green plants. These similarities in the photosynthetic mechanisms have caused the even closer relationship of the blue-green algae to bacteria to be overlooked. However, evidence has accumulated over the past few years which indicates that the blue-green algae and bacteria are very much a coherent group. This relationship has been confirmed on the basis of their nucleic acid biochemistry, fine structure, and cell wall chemistry [3—5]. Based on these similarities, Stanier et al. [3] have proposed that blue-green algae be considered as another major group of photosynthetic bacteria, called the cyanobacteria.

This procaryotic nature of the blue-green algae would seem to make them an excellent experimental organism for photosynthesis. They lack membrane-bound organelles such as nuclei and mitochondria and, thus, the photosynthetic lamella are the only intracellular membranes. This also means that the information for the photosynthetic apparatus must be coded for on only one genome. Therefore, they would seem to be well suited for the study of photosynthetic genetics. We have recently isolated temperature-sensitive mutations of the unicellular blue-green alga, *Synechococcus cedrorum*, which are defective in photosynthesis when grown at high temperature [6]. Since many of these mutants have defects in the photosynthetic membranes, a complete characterization of the aberration should include a careful study of the physical and functional parameters of the Photosystems. The search for an isolation procedure for the two photosystems in the blue-green algae is the basis of this report.

Fractionation of photosynthetic membranes by detergent or mechanical treatment and subsequent purification of the individual photosystems has become a classical tool for the study of reaction center properties [7—9]. These methods have been used to isolate small, photoactive Photosystem (PS) I particles from plants, eucaryotic algae, and blue-green algae [10—13], which appear to be free of contamination from PS II. On the other hand, purification of PS II reaction centers has been a more difficult problem. In recent years, however, small particles that contain only the reaction center of PS II have been isolated from plants and eucaryotic algae [14—16]. Unfortunately, no such active PS II particle has been isolated from the blue-green algae, despite numerous attempts [1,17—19]. Since we would expect that many of our most interesting mutants would be at or near PS II, we felt it important to isolate pure PS II particles from the photosynthetic lamellae of blue-green algae.

We report the isolation of a pure PS II particle using a digitonin fractionation procedure modeled after that of Wessels et al. [16]. The broken membranes were then separated on a sucrose gradient through sedimentation in a zonal rotor and purified by DEAE-cellulose chromatography. The resulting particle lacked the capacity to evolve oxygen, but was highly photoactive in the Hill reaction using artificial donors and acceptors. An added feature of this technique was the ability to also isolate a small PS I particle which has consequently been purified and characterized.

Methods

Algal strain and culture methods. The strain used in this study was *Synechococcus cedrorum* IU1191 obtained from the Indiana University Culture Collection (now at the University of Texas). The cells were cultured in BG-11 [20] and maintained and propagated as previously described [21]. For the isolation of membrane fragments, cells were grown in 20-l carboys containing 14 l of medium. The cultures were aerated vigorously with air at 22—25°C and illuminated with General Electric FC16 T10-CW circular fluorescent lights at an intensity of 500 ft.-candle (0.5 mW/cm^2).

Preparation of membrane fragments. 42 l of cells were grown to late log phase (about $1 \cdot 10^8$ cells/ml) and pelleted in a Beckman J-21B centrifuge using the Model JCF-Z continuous flow rotor. Centrifugation was at 10 000 rev./min for 1.5—2 h at 5°C. Depending on the density of the culture, the pellet was resuspended in 300—600 ml of buffer to a concentration of 500 μg Chl/ml. The buffer contained 10 mM KCl, 20 mM Tricine, pH 7.5, and 0.4 M sucrose. The first step in the procedure, the preparation of spheroplasts, was accomplished by the addition of 1 mg/ml lysozyme (Sigma) plus 10 mM EDTA. After incubation for 3 h at 37°C with mild stirring, the spheroplasts were either used directly for assays or broken by treatment in a Braun homogenizer. Breakage was performed by diluting the cells 1 : 1 with 0.11-mm glass beads and homogenizing for 30 s. Following Braun homogenization, $MgCl_2$ was added to 15 mM and the cells were incubated with 1—10 μg/ml DNAase at room temperature for 15 min with very slow stirring. After DNA degradation, EDTA was added to give a net EDTA concentration of 5 mM; the cells were then centrifuged for 15 min at 30 000 × *g*, the supernatant discarded, and the pellet resuspended in 10 mM KCl and 20 mM Tricine, pH 7.5. The suspension was then sonicated for 1 min to break up membrane aggregates.

Digitonin was then added to the membrane suspension to yield a final digitonin/chlorophyll ratio of 15. This was accomplished by adding a 2% solution of digitonin to the membranes so that the final concentration was 500 μg Chl/ml. This generally resulted in a final digitonin concentration of 0.75%. Digitonin incubation was at 4°C and lasted approx. 16 h. Digitonin from Sigma was generally used, though Fisher digitonin also worked well.

Isolation of Photosystem particles by centrifugation. Following digitonin treatment, large membrane fragments were discarded by centrifugation at 2000 × *g* for 5 min. The resulting supernatant was again sonicated for 1 min and then layered onto a sucrose gradient. Centrifugation was performed in two different ways depending on the amount of material utilized. In the preliminary experiments, 5 ml of samples were layered onto 34 ml of a 10—30% continuous sucrose gradient and spun for 20 h at 25 000 rev./min in a Beckman SW-27 rotor. In later experiments, large quantities of the Photosystem particles were isolated by zonal centrifugation in a Beckman Ti-15 zonal rotor. The sucrose gradient was formed by loading 183 ml each of 5, 10, 15, 20, 25 and 30% (w/v) sucrose solutions into the rotor. Approx. 125 ml of the digitonin-treated membranes were then layered on top of the gradient and centrifuged at 25 000 rev./min for 36 h. In both cases, the sucrose con-

tained 10 mM KCl and 20 mM Tricine, pH 7.5, and 0.1% digitonin and centrifugation was at 5°C. Both techniques yielded three green bands which were then purified and characterized separately (Figs. 1 and 2).

Column chromatography. The individual bands were pooled and then dialyzed against Tricine/KCl buffer. At times, this solution was concentrated using an Amicon Ultrafiltration unit and PM 30 membranes and experiments were performed on the concentrated material. When further purification was desired, the bands were adsorbed onto a DEAE-cellulose anion-exchange column (Whatman DE-23). The PS particles were eluted with a 10—600 mM KCl salt gradient (in 20 mM Tricine, pH 7.5) that contained 0.05% digitonin for PS II material and 0.2% digitonin for the PS I material. At low concentrations of digitonin, the PS I particles bound tightly to the column which allowed an easy separation of the two Photosystems.

Analytical methods. Photosystem I and II activity were measured as partial reactions using artificial electron donors and acceptors. PS I activity was determined by monitoring O_2 consumed during light-driven electron transport from DPIP-H_2 to methyl viologen with a YSI Model 53 Oxygen monitor. The reaction mixture contained (in 3 ml): 0.4 M sucrose, 40 mM Tricine, pH 7.5, 20 mM KCl, 5 mM $MgCl_2$, 1 mM KCN, 4 mM NH_4Cl, 0.1 mM methyl viologen, 0.1 mM 2,6-dichlorophenolindophenol (DPIP), 2 mM ascorbate, 10^{-5} M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), and material containing 10—30 μg Chl/ml. PS II activity was measured as the photoreduction of ferricyanide [22] with semicarbizide as donor or the reduction of DPIP with diphenylcarbazide as donor [23,24]. Ferricyanide reduction was monitored at 420 nm ($\epsilon = 0.9\ mM^{-1} \cdot cm^{-1}$) while DPIP photoreduction was measured at 590 nm, ($\epsilon = 16\ mM^{-1} \cdot cm^{-1}$). In all cases, saturating illumination was provided by quartz-halogen lights. Chlorophyll concentrations were determined by extraction in 80% acetone [1] and protein was measured by the Lowry technique [25].

Absorption and fluorescence spectra. Absorption spectra were obtained on an Aminco-Chance DW-2 dual wavelength spectrophotometer equipped with a low-temperature attachment. To calculate the relative concentration of chlorophyll and phycocyanin, the simultaneous equations of Jones and Myers [26] and Arnon et al. [1] were used with the absorbances at 678 and 627 nm.

Fluorescence spectra were obtained on a modified Aminco-Bowman spectrofluometer. The photomultiplier tube was an Hamamatsu R-666-S, a GaAs (CS) cathode-type tube, which has nearly flat response from 400—900 nm. This was connnected to a Keithley 240 high-voltage power supply and a Keithley 414S Picoammeter; the curves were plotted on an Hewlett-Packard X-Y recorder and are shown uncorrected. Fluorescence measurements were performed at 77 K in 55% glycerol.

Cytochrome and P-700 determinations. Chemically reduced minus oxidized difference spectra were performed on the DW-2 spectrophotometer using the techniques of Henningsen and Boardman [27]. Particles at a concentration of approx. 50 μg Chl/ml were suspended in a buffer containing 40 mM Tricine, pH 7.5, and 10 mM $MgCl_2$. Ferricyanide and hydroquinone were used at final concentrations of 2 and 5 mM, respectively, while dithionite was added as a solid.

The chemical oxidation of *P*-700 was obtained from dithionite minus ferricyanide difference spectra at 703 nm using an extinction coefficient of 64 $mM^{-1} \cdot cm^{-1}$ [28]. The photooxidation of *P*-700 was performed using the conditions of Shiozawa et al. [12] with 725 nm as a reference wavelength. The reaction conditions were the same as those used for cytochrome determination. The signal was measured in the presence of 2 mM ascorbate and 100 μM methyl viologen. The actinic light was passed through a Baird-Atomic 440 nm interference filter, while a Corning CS2-64 blocking filter was placed before the photomultiplier.

Gel electrophoresis. The isolated photosystems were analyzed by SDS acrylamide gel electrophoresis as described in detail previously [29,30]. The samples were run on a slab gel apparatus similar to that first described by Studier [31]. The analyzing gel contained a gradient of 10–15% acrylamide with the SDS buffer system of Laemmli [32]. Samples were treated with 1% SDS and 5% β-mercaptoethanol, boiled for 1 min, and then run for about 4 h at 16 mA. Molecular weights were obtained by co-electrophoresis of the following proteins: phosphorylase *a* (92 000), bovine serum albumin (67 000), ovalbumin (45 000), chymotrypsinogen A (25 000) and cytochrome *c* (12 500).

Electron microscopy. The particles were negatively stained with 1% uranyl acetate for 1 min and air-dried. Micrographs were taken on a Jeolco 100B microscope at 100 kV and a magnification of ×60 000.

Results

Isolation of Photosystem particles

The main goal of this work was to determine a straightforward method for the isolation of purified PS II particles. Therefore, numerous techniques were attempted before the procedure to be described was obtained. Detergents such as Triton X-100 [12], lauryl dimethyl amine oxide, SDS [33], as well as guanidine-HCl were first tried. Pure PS I fragments similar to those previously described [9,34,35] were isolated by these methods, but these chemicals appeared to destroy PS II activity. However, use of the detergent digitonin in a procedure modeled after that of Wessels et al. [16] permitted the isolation of a highly active PS II fragment as well as a purified PS I particle.

The detailed description of the procedure is given in Methods and the major steps in the protocol are listed in Fig. 1. There are two critical steps in the early stages of the isolation: Braun homogenization and digitonin treatment. We determined that homogenization in the Braun for 30 s with a 1 : 1 ratio (v/v) of glass beads to cells resulted in optimal PS II activity. The relative concentration of digitonin was even more significant; though PS II particles could be obtained at digitonin : chlorophyll ratios as low as 5 : 1, the yield of the PS II, and especially the PS I, fragments was reduced radically. Likewise, an overnight treatment with digitonin was found to be essential, and treatments of less than 6 h resulted in low yields of the Photosystem fragments. Cellular growth phase and cell stability were probably the most important factors for a sucessful isolation. The highest yields and most consistent separations were obtained with cells grown to late-log phase (approx. $1 \cdot 10^8$ cells/ml) which were broken immediately after harvesting.

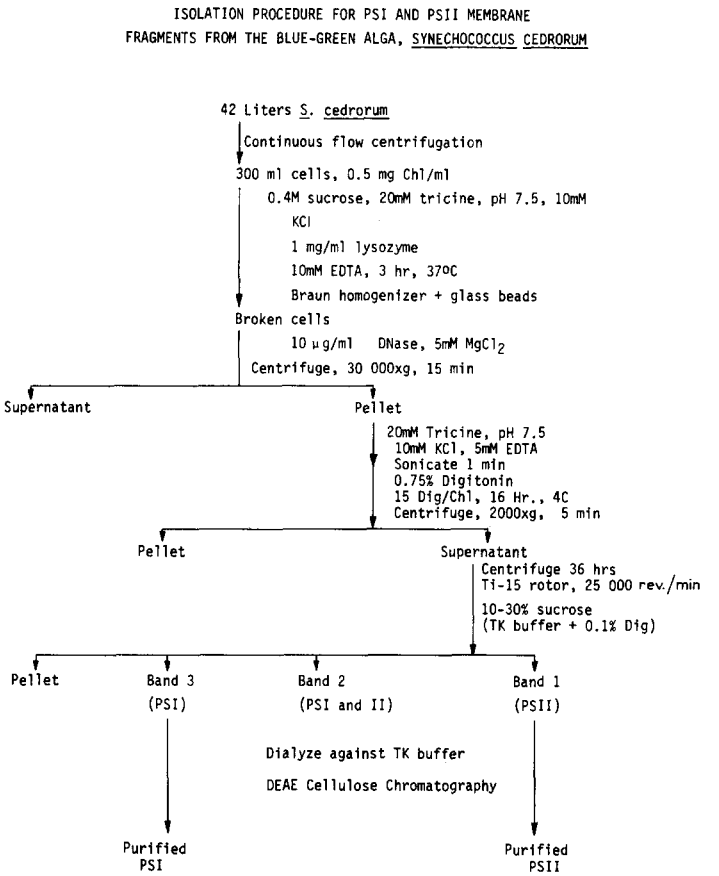

Fig. 1. Isolation procedure for PS I and PS II membrane fragments from the blue-green alga, *S. cedrorum*. Dig, digitonin; TK buffer, Tricine/KCl buffer.

TABLE I

GENERAL PROPERTIES OF PS-I AND PS-II MEMBRANE FRAGMENTS FROM SW-27 ROTOR SUCROSE GRADIENTS

The basic features of the three discrete bands obtained by sedimentation of digitonin-treated membranes through 10—30% sucrose for 20 h at 27 000 rev./min. The data from the room temperature absorption spectra were treated by the simultaneous equations of Arnon et al. [1] to obtain a molar ratio of Chl *a* to phycocyanin (phy). The Chl : *P*-700 ratio was determined from a dithionite minus ferricyanide difference spectra. The rates of photosynthesis were performed as described in Methods. SC, semicarbazide; MV, methyl viologen.

Gradient position	A_{629}	A_{678}	A_{629}/A_{678}	Chl *a* : Phy (nmol/ml)	Chl : Phy (molar)	Chl : *P*-700	Rate **	
							PS II (SC → FeCN)	PS I (DPIP-H_2 → MV
Band 1	0.13	0.50	0.26	8.3	0.021	395	200—600	500	80
Band 2	0.22	0.72	0.305	11.6	0.4	29	140	330	175
Band 3	0.105	0.42	0.25	6.2	n.d. *	600	90	90	350

* n.d., not detectable.

** µmol/mg chlorophyll per h.

When the digitonin-treated membranes were layered onto a 10—30% sucrose gradient, and centrifuged for 20 h in the SW27 rotor at 25 000 rev./min, five pigmented bands could be discerned. Nearest to the top was a deep blue band which contained mostly phycocyanin (λ_{max} = 620 nm). Directly underneath the phycocyanin was a greenish-brown layer that consisted of cytochromes and some chlorophyll-containing material. As will be discussed later, this layer sometimes exhibited varying amounts of PS I activity. The most important layers, which are designated (from top to bottom) bands 1, 2 and 3, sedimented reproducibly in the middle third of the gradient. Bands 1 and 3 were green, while band 2 was a bluish-green, indicating a significant quantity of phycocyanin.

The general properties of the three green bands are given in Table I. Spectral analysis at room temperature showed that the fractions differed both in their chlorophyll absorption maxima as well as in the ratio of chlorophyll to phycocyanin. The λ_{max} for the three bands were: band 1, 674—675 nm; band 2, 676 nm; band 3, 677—678 nm. The raw absorption data were treated by the simultaneous equations of Arnon et al. [1], to obtain a molar ratio of Chl *a* to phycocyanin. As shown in Table I, the results indicate that bands 1 and 3 are nearly devoid of phycocyanin, while band 2 still retains some accessory pigments. Phycocyanin in band 3 was usually below the limit of detection by this method.

All of the fractions were devoid of the capacity to evolve oxygen, but retained relatively high levels of PS I and PS II activity using artificial donors and acceptors. Bands 1 and 3 were enriched in PS II and PS I activity, respectively, while band 2 contained both activities. The fractions also differed as to the number of Chl molecules per *P*-700 as deduced from chemically reduced-minus-oxidized absorption spectra. Band 1 contained only small amounts of *P*-700, with a Chl : *P*-700 ratio ranging between 200 and 600. Band 2 showed an intermediate Chl : *P*-700 ratio of 140, while the ratio of band 3 (Chl : *P*-700 = 90) indicated that this fraction was enriched in PS I. Taken together, these results indicate that band 1 is highly enriched in PS II, band 2 more closely resembles the original membranes, while band 3 is enriched in PS I.

The purification of the Photosystem fragments was then continued by column chromatography. However, in order to obtain enough material for further characterization, material from 25—30 SW-27 tubes had to be pooled. Therefore, the isolation procedure was scaled up by sedimenting the digitonin-treated cells through a sucrose gradient in a Ti-15 zonal rotor. Besides giving much greater yields, this technique also allowed an even better separation of the Photosystems. An analysis of the Photosystem activity after zonal sedimentation is shown in Fig. 2. The overall pattern was the same as that observed after sedimentation in the swinging-bucket rotor; there were three chlorophyll peaks (data not shown) with most of the phycocyanin remaining near the top of the gradient. The PS II activity was mostly concentrated between fractions 20—40 (band 1) with a much smaller peak around fraction 45. Photosystem I activity was mostly confined to fractions 50—60, though fairly high activity is noticeable in the band 2 region. In some experiments, a large quantity of PS I activity was apparent at the top of the gradient. We will discuss this feature later.

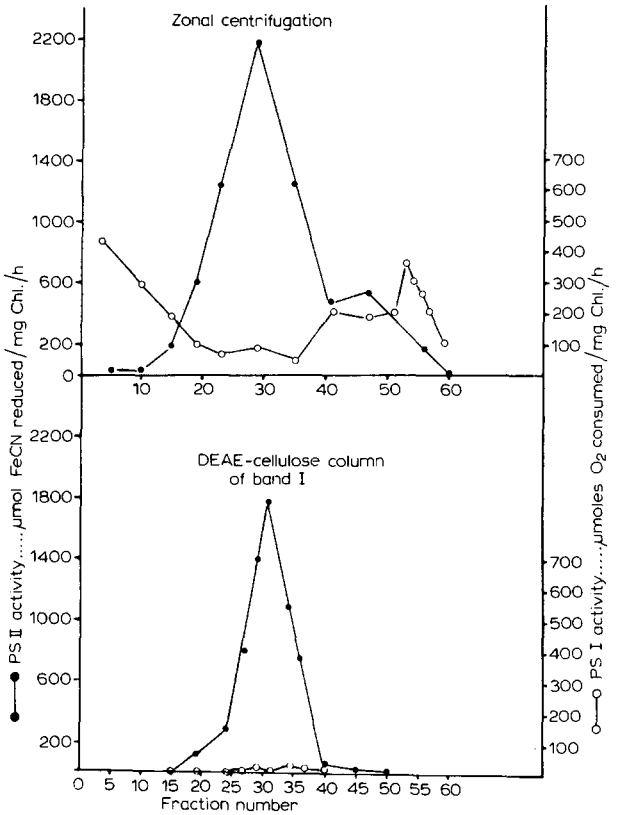

Fig. 2. (A) Zonal centrifugation of digitonin-treated membranes. A 1200 ml sucrose step gradient of 10—30% sucrose was loaded into a Beckman Ti-15 Zonal rotor, followed by 125 ml of sample. The material was centrifuged at 25 000 rev./min for 36 h at 4°C; 60 fractions of about 20 ml each were collected and monitored for PS I and PS II activity. (B) The material from fractions 24—34 in the zonal run in (A) were pooled, desalted and run on a DEAE-cellulose column equilibrated with 10 mM KCl, 20 mM Tricine, pH 7.5, and 0.05% digitonin. The material was eluted with a 10—60 mM KCl gradient, fifty 1-ml fractions were collected and the Photosystem assays performed.

The peak fractions were then pooled (fractions 25—35 for PS II and 50—58 for PS I) and purified further by means of column chromatography. In our initial studies we tried a variety of different support media and found DEAE-cellulose to be the most useful. Hydroxyapatite, which has proven to be bene-

TABLE II

PURIFICATION OF PHOTOSYSTEM II MEMBRANE FRAGMENTS

SC, semicarbazide; MV, methyl viologen; Phy, phycocyanin.

Material	Photosynthetic rate (μmol/mg chlorophyll per h)		
	PS II (SC → FeCN)	PS I (DPIP-H_2 → MV)	Chl : Phy (molar)
Spheroplasts	500	800	2.3
Zonal; peak fraction	2179	80	380
DEAE column eluate; peak fraction	3150	20	472

ficial for purification of many plant and algal Photosystem preparations [9,10, 16], had only a marginal effect on these fragments. Elution on DEAE-cellulose was simplified by the different physical properties of the PS I and PS II fragments. The PS II particle could be eluted with only 0.05% digitonin in the buffer; under these conditions PS I activity was bound to the column. Hence, the purification of PS I fragments required high concentrations (0.2% or higher) of digitonin in the elution buffer to prevent the material from being irreversibly adsorbed to the column.

The results of chromatography of the band 1 material on a DEAE-cellulose column is graphically depicted in Fig. 2B, while the total purification of the PS II particle from another experiment is summarized in Table II. It is obvious that this material is highly enriched in PS II activity and nearly devoid of PS I. The spectra indicate that trace amounts of phycocyanin may still be present even after chromatography. However, such quantities are at the maximum level of sensitivity of the equations and may not be significant. An interesting aspect of Table II is that the material from the zonal rotor itself is very pure. Therefore, some of the experiments to be reported on later used material directly from the zonal run, which was then desalted and concentrated. Similar results were obtained for the PS I particles (Newman, P.J., and Sherman, L.A., unpublished).

Properties of the PS II particle

The photochemical activity of the PS II fragments was measured either as the reduction of ferricyanide using semicarbazide as electron donor or the photoreduction of DPIP with diphenylcarbazide as donor. Material from the zonal rotor generally had activities of 2000 μmol/mg Chl per h with ferricyanide and 50—70 μmol/mg Chl per h with DPIP. After DEAE-cellulose chromatography, these values increased to about 3000 and 70—100, respectively. The rather low activity with DPIP is typical with these cells, since we usually obtain values of only 50—100 μmol/mg Chl per h with fresh spheroplasts under our conditions. Most importantly, both activities are DCMU-sensitive (Table III). DPIP photoreduction is inhibited 75% at 30 μM DCMU,

TABLE III

DCMU SENSITIVITY OF PHOTOSYSTEM II ACTIVITY OF PS II MEMBRANE FRAGMENTS

Light-induced Photosystem II activity was measured as the photoreduction of ferricyanide (FeCN) or dichlorophenolindophenol (DPIP) using semicarbazide (SC) or diphenylcarbazide (DPC), respectively, as electron donors. DCMU was added from a 10^{-3} M solution in ethanol and 0.15 ml ethanol was added to the control. Reaction conditions are presented in Methods.

Addition of DCMU (μM)	SC → FeCN (μmol/mg Chl per h)	Inhibition (%)	DPC → DPIP (μmol/mg Chl per h)	Inhibition (%)
0	2150	0	130	0
10	1270	41	60	54
25	1050	51	—	—
30	—	—	28	78
50	510	76	14	89

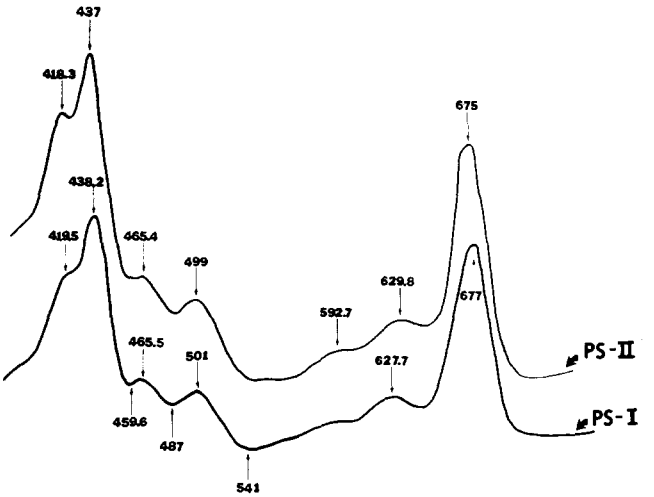

Fig. 3. Liquid N_2 absorption spectra of the PS I and PS II particles obtained by digitonin-treatment and zonal centrifugation.

while ferricyanide reduction is inhibited to this extent at a DCMU concentration of 50 μM.

Spectral properties

The liquid nitrogen absorption spectra of the Photosystem particles (Fig. 3) indicate that the absorption maximum for PS II is located at 675 nm, while that of PS I is at 677 nm. The peaks near 629 nm imply that phycocyanin is present in very low quantities; using the equations of Arnon et al. [1], the molar ratio of Chl : phycocyanin is always greater than 400. The peaks near 465 and 499 nm indicate that both particles contain carotenoids, mainly β-carotene.

The liquid nitrogen fluorescence spectra of various zonal fractions are shown in Fig. 4. Fraction 28, which contains the peak of the PS II activity (Fig. 4A), has a near symmetrical spectrum with λ_{max} = 685 nm. This result is similar to that obtained recently with other purified PS II particles [14,16]. As we go towards the bottom of the gradient and into an area of higher PS I activity, we get an increase in fluorescence at 696 nm. By fraction 45, which is the peak of band 2, the 696 nm fluorescence is approximately equal to that at 686 nm and a prominent shoulder at 715 nm appears (Fig. 4D). Fraction 52, which has predominantly PS I activity, shows only a peak at 715 nm, with a shoulder at 700 nm. Again, this is similar to other purified PS I particles that have been isolated from algae [11,17,18]. After the PS II material was pooled and run twice on a DEAE-cellulose column, the fluorescence spectrum shown in Fig. 4F was obtained. The peak is still at 685 nm, but there is now no trace of a shoulder at 696 nm. These results imply that fluorescence at 685 nm is indicative of PS II, that fluorescence at 696 nm is prominent when both Photosystems are present, and that fluorescence at 700—715 nm is representative of PS I activity. The close relationship of Fig. 4A and F also indicates that the peak fractions directly from the zonal run are already highly purified and may not require further purification for many studies.

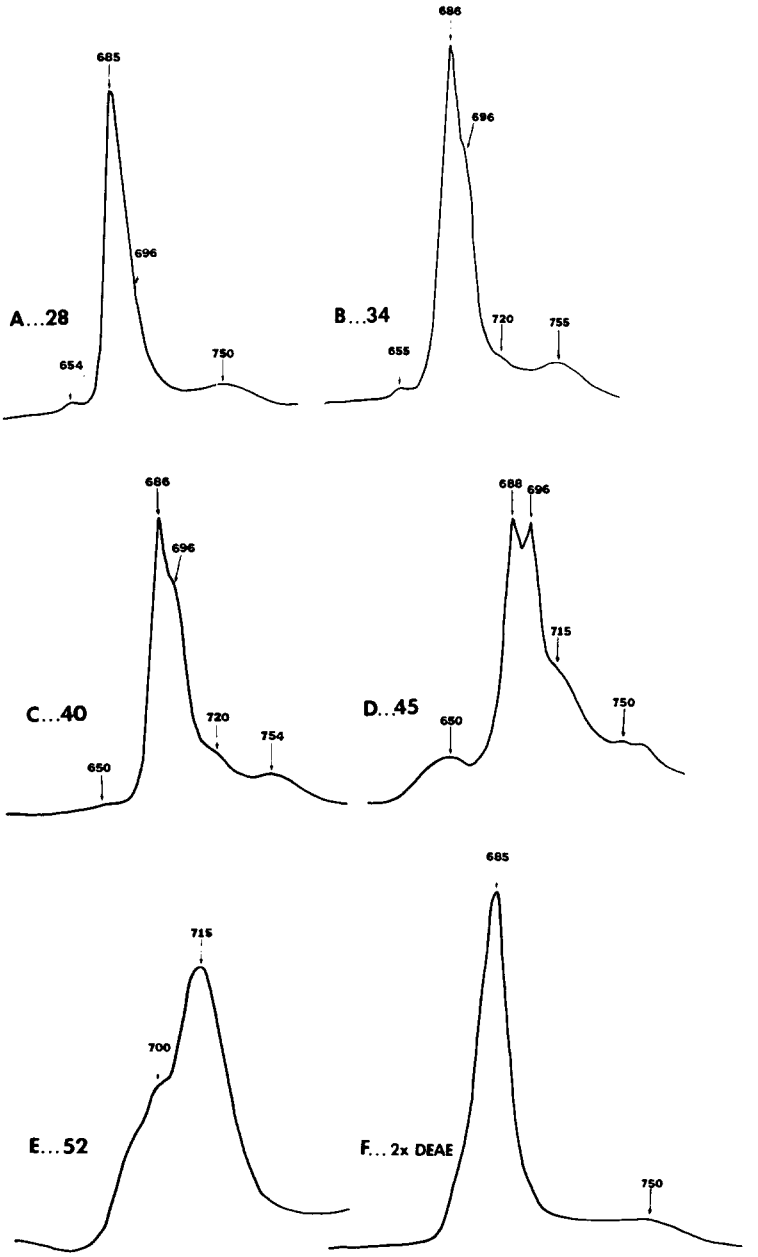

Fig. 4. Liquid N_2 fluorescence spectra of selected zonal fractions containing 55% glycerol and 10 μg Chl/ml. (A) Fraction 28, the peak of the PS II material; (B) Fraction 34, from the trailing edge of band 1; (C) and (D) Fractions 40 and 45 from the band 2, material containing PS I and PS II material; (E) Fraction 52, the peak of the PS I material; (F) Pooled band 1 material after consecutive elution from 2 DEAE-cellulose columns.

Cytochrome content

The cytochrome content of the particles was determined from chemically reduced-minus-oxidized difference spectra as shown in Fig. 5 for the PS II

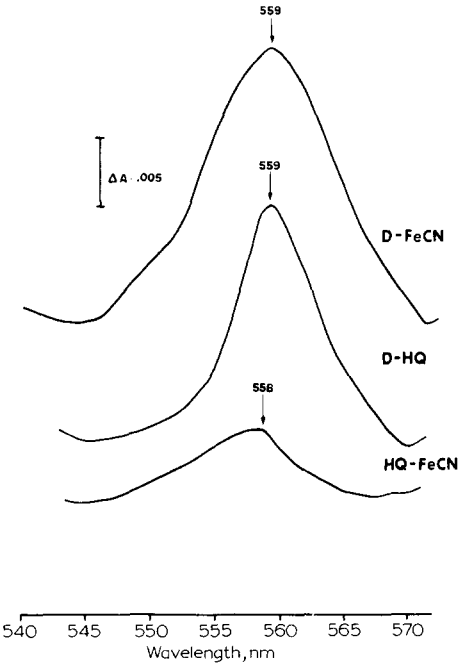

Fig. 5. Chemically oxidized-minus-reduced difference spectra of the PS II fragment purified by DEAE-cellulose chromatography. Hydroquinone (HQ) and ferricyanide (FeCN) were added to a final concentration of 2 mM, while dithionite (D) was added as a solid. The reaction mixture contained: 0.4 M sucrose, 20 mM Tricine, pH 7.5, 10 mM $MgCl_2$ and particles to 40 μg Chl/ml.

particles. The top curve represents the dithionite reduced-minus ferricyanide-oxidized spectrum that yields information on the total cytochrome *b* content [27]. It is apparent that the main cytochrome *b* is cytochrome *b*-559 with only trace amounts of cytochrome b_6. That the cytochrome *b*-559 is mostly in the low-potential form is indicated by the hydroquinone reduced-minus ferricyanide-oxidized spectrum. This spectrum shows a very low quantity of cytochrome *f* and a small peak at 558 nm. The dithionite-minus-hydroquinone spectrum is also indicative of a low cytochrome b_6 content. These results are summarized in Table IV. On average, the PS II particles contain one cyto-

TABLE IV

CYTOCHROME AND *P*-700 CONTENT OF PURIFIED PS I AND PS II MEMBRANE FRAGMENTS

The ratio of Chl to cytochromes and *P*-700 were obtained from chemically reduced-minus-oxidized difference spectra. The techniques were basically those of Henningsen and Boardman [27]. D — FeCN, dithionite minus ferricyanide; D — HQ, dithionite minus hyroquinone; HQ — FeCN, hydroquinone minus ferricyanide. Conditions were those of Fig. 5. Average values are given in parentheses.

Particle	Chl : cytochrome *b*-559 (D — FeCN)	Chl : cytochrome *b*-559 LP (D — HQ)	Chl : cytochrome *b*-559 HP (HQ — FeCN)	Chl : cytochrome *f* (HQ — FeCN)	Chl : *P*-700 (D — FeCN)
PS II	38—66 (50)	40—70 (55)	1000	525	400
PS I	800	—	—	—	40

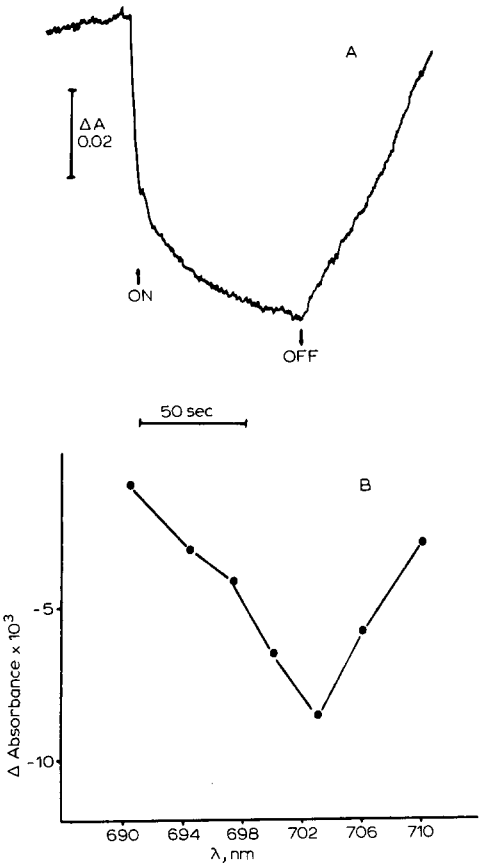

Fig. 6. Oxidation of *P*-700 in the PS I particle after zonal centrifugation. The particles were suspended in the buffer listed in Fig. 5 at a concentration of 50 μg Chl/ml, to which 2 mM ascorbate and 0.1 mM methyl viologen were added. (A) The kinetics of the absorbance chance at 703 nm. (B) The *P*-700 spectrum between 690—710 nm using 725 nm as a reference.

chrome *b*-559 per 50 chlorophylls of which no more than 10% are in the high-potential form. Only trace amounts of cytochrome *f* are in the PS II particles and trace quantities of cytochrome *b*-559 in the PS I particle.

P-700 activity

The two particles differ greatly with respect to their *P*-700 activity. The PS II particles contain only minor quantities of *P*-700, averaging one *P*-700/400 chlorophyll molecule as determined by dithionite-ferricyanide difference spectra (Table IV). However, the PS I particle is highly enriched for *P*-700. The light-induced difference spectra for the PS I fragment (Fig. 6) has an absorption maximum at 703 nm. Quantitation of chemically-induced difference spectra yield a Chl : *P*-700 ratio as low as 40 after DEAE-cellulose chromatography (Table IV).

Protein composition

The protein composition of the purified PS I and PS II fragments is dis-

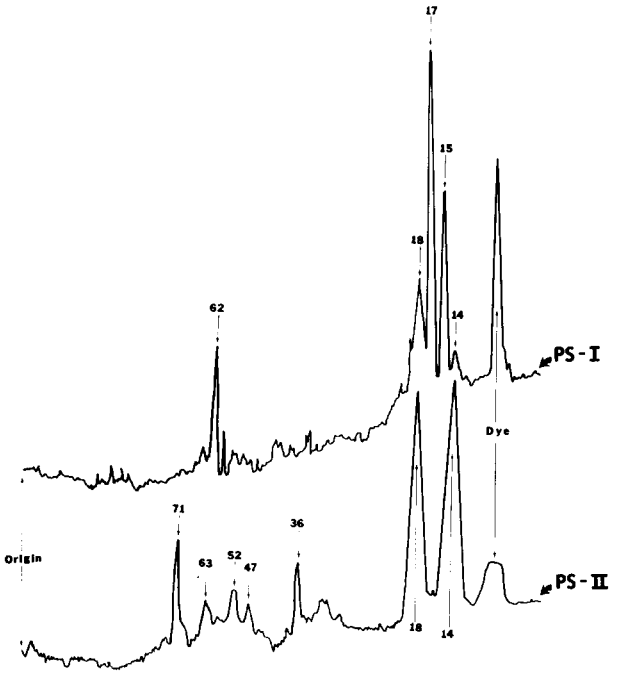

Fig. 7. Densitometer scans of SDS acrylamide gels containing the PS I and PS II particles. The gels were 10—15% acrylamide gradients which utilized the buffer system of Laemmli [32]. The numbers above the peaks are the molecular weight expressed in kilodaltons.

played in Fig. 7. This figure shows densitometer scans of 10—15% acrylamide gradient gels after the particles had been denatured with SDS and β-mercaptoethanol. The results of these gels are summarized and quantitated in Table V. The PS II particle consists of seven proteins, ranging in molecular weight from

TABLE V

PROTEIN COMPOSITION OF PS I AND PS II PARTICLES

The densitometer scans in Fig. 7 were traced, the peaks cut and then weighed. The percentage composition was obtained by dividing the weight of each individual peak by the total weight. This figure was multiplied by 10^6 dalton and then divided by the molecular weight of each species. The values are rounded off to the nearest integer. For the PS I particle, these values were then divided by 3 to yield the smallest particle which contains one copy of the 62 000 dalton protein.

Molecular weight	PS II particle		Molecular weight	PS I particle	
	Composition (%)	Copies/10^6 dalton		Composition (%)	Copies/$3.3 \cdot 10^5$ dalton
71 000	10.2	1			
63 000	5.6	1	61 000	17	1
52 000	8.1	2	18 000	27	5
47 000	4.5	1	17 000	29	5
36 000	6.9	2	15 000	21	5
18 000	33	18	14 000	6	1
14 000	32	23			

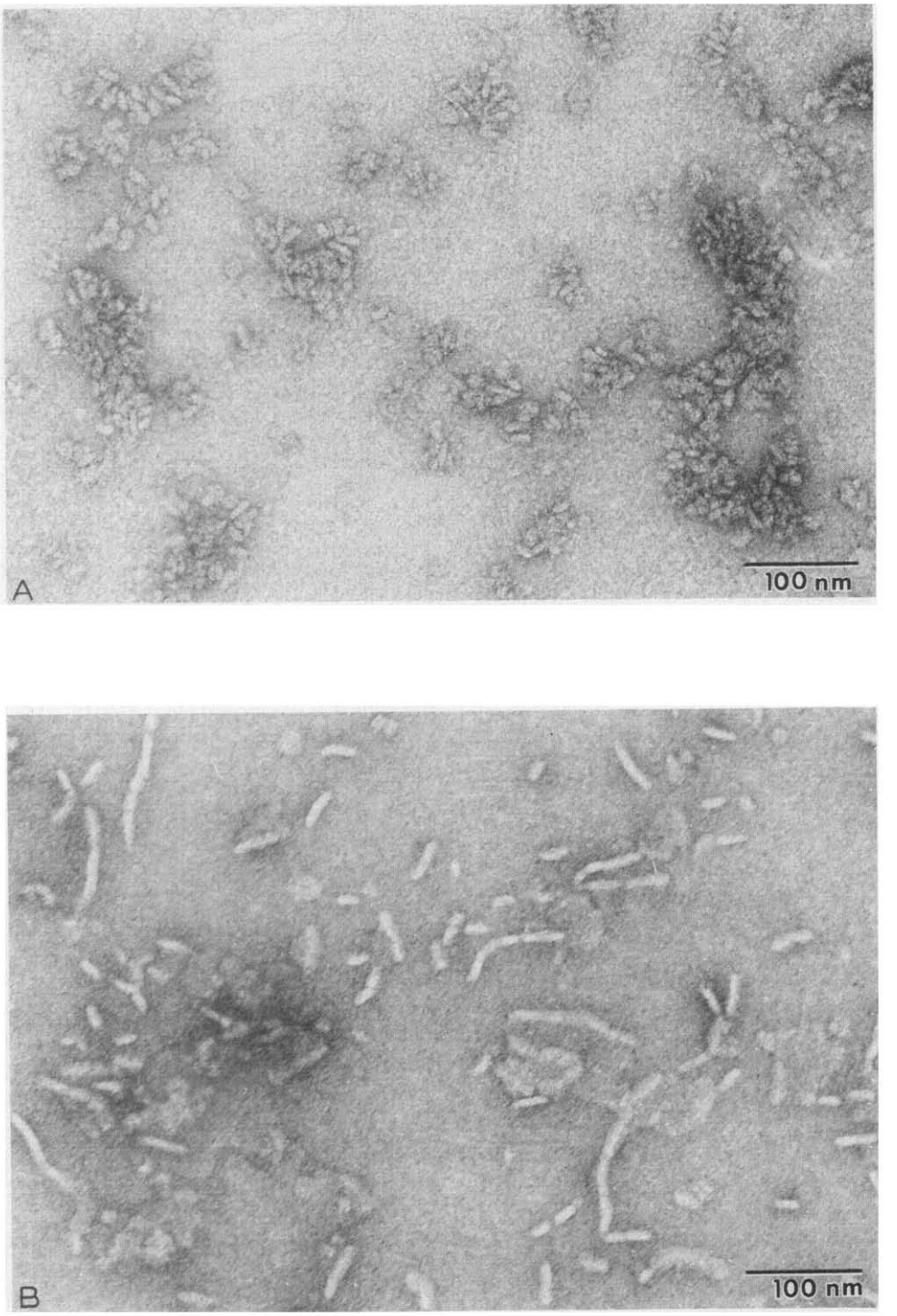

Fig. 8. Electron micrographs of the purified PS II (top) and PS I (bottom) particles stained with 1% uranyl acetate.

71 000 to 14 000. In some of the zonal fractions, another prominent band at about molecular weight 12 000—13 000 appears, but its appearance was variable and will not be considered here. By calculating the percentage composition of each band, it was possible to compute the relative number of copies of each protein in the PS II particle. When such a calculation is made, the lowest molecular weight that allows an integral value for each protein is $1 \cdot 10^6$ (Table V). Of course, if any of the heavier proteins are contaminants or not part of the reaction center, this value could drop appreciably. However, this exercise does indicate that the 14 000 and 18 000 proteins constitute at least 65% of the PS II particle. We will discuss the relationship of the lower molecular weight proteins to the heavier proteins in Discussion.

The PS I particle would appear to be somewhat less complex with five proteins of molecular weight 62 000, 18 000, 17 000, 15 000 and 14 000. The rather low quantity of the 14 000 species could imply that it is a PS II contaminant. Utilizing the same calculations, a PS I particle of approx. $3.3 \cdot 10^5$ dalton could be composed of one molecule of the 62 000 (and 14 000) dalton protein and five molecules each of the 18 000, 17 000 and 15 000 dalton proteins.

Electron microscopy

The structure of the two particles, as determined by electron microscopy of negatively stained preparations, is shown in Fig. 8. The PS II fragment is a homogeneous, rod-shaped particle with dimensions of 18 × 6 nm. The material has a slight tendency to aggregate and there is a background of somewhat smaller particles. The PS I fragment is also a rod, but with an average axial ratio of 7 : 1 (44 × 6 nm). This particle has a heterogeneous size distribution, with a number of long rods appearing to be in the process of breaking. The size and shape of the particles correspond well with their positions in the sucrose gradient and may be helpful in explaining the action of digitonin on the membranes of blue-green algae.

Discussion

We have shown that digitonin treatment of membranes from the blue-green alga, *S. cedrorum*, followed by zonal centrifugation and DEAE-cellulose chromatography can allow the isolation of fractions highly enriched in either PS I or PS II. The PS II particle shows high rates of photoreduction of certain Hill acceptors when supplied with suitable electron donors; furthermore, this activity is DCMU-sensitive. This particle is typified by a low Chl : cytochrome *b*-559 ratio, a low-temperature fluorescence maximum at 685 nm, and contains seven polypeptides. The PS I particle is highly enriched in *P*-700, has a low cytochrome content, a fluorescence maximum at 715 nm, and contains five protein species.

The nature of digitonin fractionation of blue-green algal membranes is not known, but it must obviously differ in some respects from its action in plants. Evidence has accumulated in recent years that the primary effect of digitonin is to separate stroma lamellae, containing only PS I, from the grana lamellae containing both PS I and II [7—10]. The best separations are achieved with chloroplasts that have grana and which are suspended in high salt solution to

prevent disruption of the grana. Nonetheless, digitonin acts on the grana membranes as well, and can cause a separation of grana PS I and PS II [36,37].

Since the blue-green algae contain no stacked membranes, it is obvious that we must look elsewhere for a mechanism. It appears that in blue-green algae the small Photosystem II particles are first removed, leaving a membrane matrix which contains most of the PS I activity. The remaining membrane can continue to fragment, ultimately producing very small PS I particles that band near the top of the gradient (see Figs. 2 and 8). The initial release of PS II fragments does not necessarily imply that PS II is on the external surface of the membrane. It simply implies that membranes from cells broken in the Braun homogenizer are a good substrate for digitonin-mediated release of PS II particles from the membrane.

Using the filamentous blue-green alga, *Nostoc muscorum*, Arnon et al. [1] were not able to separate the Photosystems using digitonin, even with an 18 h treatment. However, their procedure differed from ours in many respects, so that meaningful comparisons are hard to make. We have found that the critical parts of the isolation include: the physiological state of the cells, lysozyme treatment, Braun homogenization, prolonged digitonin treatment at fairly high digitonin : Chl ratios, and sucrose gradient centrifugation. With a procedure similar to that of Arnon et al. [1] we were also unable to separate Photosystem activity. On the other hand, we are not able to retain high rates of photosynthetic electron transport from H_2O to $NADP^+$ either before or after digitonin treatment. These facts may reflect on some substantial differences which exist between filamentous and unicellular blue-green algae [3,38].

A number of Photosystem II particles have been isolated from chloroplasts in recent years. In particular, the Triton-prepared particles studied by Vernon et al. [39] and Ke [14,15], and the digitonin particles isolated by Wessels et al. [16], resembles these blue-green algal PS II particles in many respects. The photochemical activity, absorption and fluorescence spectra, and Chl : cytochrome *b*-559 ratio are all similar. The three particles contain cytochrome *b*-559 primarily in the low potential form, which may be a result of the detergent converting the cytochrome from a high to a low potential form [16,40]. Cytochrome *b*-559 is essentially the only cytochrome present in the particles.

The nature of the emission from PS I and PS II has been heavily studied for many years [41]. The low-temperature emission spectrum of blue-green algae yields bands at 686, 696 and 715 nm [41], while in chloroplasts the far red band is located around 735 nm. Based on earlier work with spinach chloroplasts and with chloroplast fragments enriched in PS II, it had been suggested that the fluorescence at 696 nm emanated from the PS II reaction center [16, 42,43]. However, both our results and recent work with chloroplasts seem to indicate that detergent-treated PS II particles with an absorption maximum at 674—675 nm fluoresce at 685 nm at 77 K. The fluorescence of band 2 is also interesting in this regard. This band fluoresces at both 686 and 696 nm and the amount of 696 nm fluorescence increases with particle size (Fig. 4, C and D). It may be that fluorescence at 696 nm is a unique property of certain chlorophylls in a specific environment; this environment may be modified or lost upon the release of the individual Photosystems by the different detergents.

The properties of our PS I particle also resemble those recently isolated

from chloroplasts and algae [9,10,12,13,34,44]. The similarities include absorption and fluorescence spectra, as well as *P*-700 and cytochrome content. The *P*-700 spectrum with a minimum at 703 nm (Fig. 7), resembles that obtained by Knaff [45] using membrane fragments of the blue-green alga, *N. muscorum*. We have not yet investigated PS I activity by photoreduction of $NADP^+$, but have used light-induced O_2 consumption during electron transport from DPIP-H_2 to methyl viologen as our PS I assay. The somewhat low PS I activity is most likely due to the known effect of high digitonin : chlorophyll ratios on this reaction [46].

The protein composition of both Photosystem particles is simple and is dominated by low molecular weight proteins (Fig. 7 and Table V). The main subunits in the PS II particle have molecular weights of 18 000 and 14 000, while those of PS I have molecular weights of 18 000, 17 000 and 15 000. Based on the relative distribution, it is possible that the 63 000 dalton protein in PS II and 14 000 dalton protein in PS I are contaminats from the other Photosystems. The computation performed in Table II indicates that PS I may have a molecular weight of no greater than 330 000 based on one 62 000 protein per unit. This would correspond closely to the model proposed by Thornber and co-workers [10—12]. Using PS I particles prepared by SDS or Triton-treatment, they obtained similar Chl : *P*-700 ratios to those reported here. On the basis of molecular size, Chl : *P*-700 ratio and chlorophyll : protein ratio, they proposed that their preparations contained three 110 000 dalton subunits, only one of which contained *P*-700. Each subunit was thought to contain 14 molecules of Chl *a*, yielding a Chl : *P*-700 ratio of about 40. A quite small PS I particle has also been obtained by Bengis and Nelson [13,44] by digitonin treatment. This particle contains six proteins, of which only the 70 000 dalton species is needed for *P*-700 oxidation. It may be that the 62 000 dalton protein described here and the 70 000 dalton protein of Bengis and Nelson [13,44] have related functions. Such a possibility is under investigation.

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