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PHOTOCHEMICAL ACTIVITY AND COMPONENTS OF MEMBRANE PREPARATIONS FROM BLUE-GREEN ALGAE

I. COEXISTENCE OF TWO PHOTOSYSTEMS IN RELATION TO CHLOROPHYLL *a* AND REMOVAL OF PHYCOCYANIN

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SUMMARY

Nostoc muscorum (Strain 7119) cells were disrupted and the accessory pigment phycocyanin was removed from membrane fragments by digitonin treatment. The phycocyanin-depleted membrane fragments retained both Photosystem I and Photosystem II activity, as evidenced by high rates of $NADP^+$ photoreduction either by water or by reduced 2,6-dichlorophenolindophenol, indicating that phycocyanin is not an essential component for electron transport activity.

No separation of the two photosystems was effected by the digitonin treatment. Even drastic digitonin treatments failed to diminish significantly the remarkably stable electron transport from water to $NADP^+$.

Action spectra and relative quantum efficiency measurements demonstrated the existence of both Photosystem I and Photosystem II in membrane fragments which contained chlorophyll *a* as the only significant light-absorbing pigment.

INTRODUCTION

Blue-green algae are procaryotic cells so similar in several fundamental respects to bacteria that Stanier et al. [1] have recently proposed that they be declassified as algae and recognized as another major group of photosynthetic bacteria, i.e. cyanobacteria [2]. The traditional classification of these cells as algae was established when their photosynthetic apparatus and activity were recognized as being different from those of photosynthetic bacteria but similar to those of green algae and higher

Abbreviations: $DCIPH_2$, reduced 2,6-dichlorophenol indophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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plants. Unlike photosynthetic bacteria, blue-green algae contain chlorophyll *a* and no bacteriochlorophyll, and their photosynthetic CO_2 assimilation is accompanied by O_2 evolution. Blue-green algae are the oldest known O_2-evolving photosynthetic cells, having existed on earth about $3 \cdot 10^9$ years [3, 4].

The photosynthetic apparatus of blue-green algae, which may be viewed as a precursor of the more highly evolved type found in chloroplasts, includes membrane structures that contain bound chlorophyll *a* (but no chlorophyll *b*), *β*-carotene, cytochromes, and other photosynthetic cofactors. The accessory light-absorbing pigments consist of C-phycocyanin, allophycocyanin, and sometimes phycoerythrin [5, 6]. These phycobiliproteins (of which C-phycocyanin usually accounts for at least 90 % by wt [2]) are soluble in aqueous solvents and are located in special organelles, phycobilisomes, that are attached to the outer surfaces of the chlorophyll-containing membranes [6].

It is widely held that in intact blue-green algal cells light directly absorbed by chlorophyll normally makes only a minor contribution to photosynthesis. Most of the light energy is first absorbed by phycocyanin and then transferred to the membrane-bound chlorophyll *a* (see [2] and literature cited therein). (The term phycocyanin will be used hereafter to denote the sum of C-phycocyanin and allophycocyanin.) In contrast to the widely recognized light-gathering role of phycocyanin in whole cells, its role in partial photosynthetic reactions, such as Hill reactions by cell-free preparations, has been a matter of some controversy. Thomas and De Rover [7] found that removal of part of phycocyanin from a cell-free preparation of *Synechoccus cedrorum* resulted in a total loss of photochemical oxygen evolution. Fredricks and Jagendorf [8] observed that an aqueous extraction of membrane fragments of *Anacystis nidulans* resulted in a loss of both phycocyanin and Hill reaction activity. Hill reaction activity was restored by the addition of the extract but on fractionation they found that the active component in the extract was not phycocyanin but another water-soluble protein [8]. However, Susor and Krogmann [9] found that Hill activity of membrane fragments (from *Anabaena variabilis*) did not depend on phycocyanin content nor on a special protein. The photoreduction of $NADP^+$ by water [10] also showed no phycocyanin requirement [11]. None of these experiments produced evidence that the photoreduction of either $NADP^+$ or the Hill reagents was, as in chloroplasts, accompanied by noncyclic photophosphorylation.

The first evidence for noncyclic photophosphorylation in blue-green algae (*Anacystis nidulans*) came from the laboratory of Trebst [12, 13]. Active and well-coupled noncyclic photophosphorylation in cell-free preparations of *Phormidium luridum* was obtained by Biggins [14, 15] through osmotic lysis of isolated protoplasts. These experiments suggested that noncyclic photophosphorylation could be demonstrated only when the cells were broken by mild techniques that would necessarily leave appreciable amounts of phycocyanin attached to the membrane fragments.

That membrane fragments (essentially free of phycocyanin) from a blue-green algae (*A. variabilis*) are capable of cyclic photophosphorylation (catalyzed by phenazine methosulfate) was first observed by Petrack and Lipmann [16] and confirmed and extended by Duane et al. [17] and Biggins [15]. Bothe [18] found that, as in chloroplasts [19], ferredoxin may serve as a catalyst of cyclic photophosphorylation in cell-free preparations of *A. nidulans*.

The work reported here is the initial part of a systematic investigation under-

taken to compare the components of the photosynthetic apparatus and the photosynthetic activity of blue-green algae with their counterparts (more advanced in the evolutionary scale) in chloroplasts. To establish maximum dependence on photochemical reactions in the blue-green algal cells and in the photosynthetic membranes isolated from them, a species (*Nostoc muscorum* Strain 7119) capable of nitrogen fixation [20] was selected and grown anaerobically in an inorganic medium with CO_2 as the sole carbon source and with N_2 as the sole nitrogen source. The photoassimilation of N_2 [21–24], like that of CO_2 [25], depends on reduced ferredoxin and ATP that photosynthetic cells form by photochemical reactions (cf. [26]).

The isolated membrane fragments had a wide range of photochemical activity, i.e. capacity for photoreduction of $NADP^+$ with water and for cyclic and noncyclic photophosphorylation. Subsequent treatments with the detergent digitonin by which phycocyanin was nearly or completely removed from the membrane fragments did not abolish their light-induced electron transport from water to $NADP^+$, which proved to be remarkably stable. In contrast to digitonin treatments of chloroplasts [27], even drastic digitonin treatments of *Nostoc* membranes did not yield fragments enriched in either Photosystem I or II. The coexistence of Photosystems I and II in membrane fragments depleted of phycocyanin and essentially dependent on chlorophyll *a* alone for light absorption was documented by determinations of action spectra and relative quantum efficiency.

METHODS

Culture of cells

The blue-green alga used, *N. muscorum* (Strain 7119) originally came from the culture collection of the University of Washington and was kindly made available to us by Dr Roger Y. Stanier. The cells were grown in multiple 10-l batches, each in a Pyrex carboy (No. 1595), continuously illuminated by a bank of tungsten filament reflector flood lamps (150 Watt). The average light intensity reaching the surface of the carboy was about 3000 lux. To prevent localized overheating of the cultures, vigorous air circulation was maintained by fixed blowers and the light was filtered through a layer of water 5 cm thick provided by a row of water-filled Pyrex Roux bottles (No. 1290). When the cells reached a high density, additional illumination was provided from the opposite side by a portable bank of fluorescent lights. The ambient temperature was kept at 20 °C. The culture medium was continuously stirred with 1.2 cm × 7.5 cm magnetic stirring bars.

The composition of the inorganic nutrient medium is given in Table I. The complete nutrient solution but with potassium phosphate omitted (to avoid possible precipitation) was sterilized by autoclaving for 45 min at 15 lb/inch^2. The potassium phosphate was autoclaved separately, cooled, and added to the cooled, sterilized nutrient solution. The final pH of the complete nutrient solution was about 8.2.

N_2 and CO_2 were continuously supplied to each carboy as a gas mixture of CO_2–N_2 (2:98, v/v). Each carboy had a rubber stopper through which were inserted glass gas inlet and outlet tubes. The gas inlet tubing was fitted with a fritted glass end that was positioned 1 to 2 cm above the level of the culture medium (a procedure of gas delivery found to be desirable with other algal cultures in our laboratory [28]). The gas outlet tubing terminated in an open test tube attached to the outside of the

TABLE I

NUTRIENT SOLUTION FOR PHOTOSYNTHETICALLY GROWN N_2-FIXING BLUE-GREEN ALGAE

Component	Amount per l	Final Concn
$MgSO_4 \cdot 7H_2O$	0.124 g	0.0005 M
$CaCl_2 \cdot 2H_2O$	0.015 g	0.0001 M
$K_2HPO_4 \cdot 3H_2O$	0.457 g	0.002 M
NaCl	0.117 g	0.002 M
Fe-EDTA *	1.0 ml	Fe, 5; K, 13 mg/l
D7, micronutrients **	1.0 ml	B, 0.5; Mn, 0.5 mg/l Mo, 0.5; V, 0.1 mg/l Zn, 0.05; Cu, 0.02 mg/l Co, 0.01 mg/l

* Fe-EDTA solution was prepared by dissolving 16 g EDTA and 10.4 g KOH in 186 ml water and mixed with an iron solution prepared by dissolving 13.7 g $FeSO_4 \cdot 7H_2O$ in 364 ml water. Air was bubbled through the mixture overnight to oxidize the iron to the ferric form. The final solution had a pH of approx. 3 and contained 5 mg Fe and 13 mg K. per ml.

** The D7 solution was prepared by dissolving in 1 l water the following: H_3BO_3, 2.86 g; $MnCl_2 \cdot 4H_2O$, 1.81 g; $ZnSO_4 \cdot 7H_2O$, 0.222 g; $CuSO_4 \cdot 5H_2O$, 0.079 g; $Na_2MoO_4 \cdot 2H_2O$, 1.26 g; $NaVO_3$, 0.239 g; and $CoCl_2 \cdot 6H_2O$, 0.0403 g.

carboy and partly filled with water. The continuity of the gas flow (approx. 20 ml/min) was visually monitored by the bubbling of the outlet gas in the test tube.

The same inorganic nutrient medium (Table I), N_2–CO_2 gas mixture, illumination, and stirring method were used for growing the inoculum for the carboy cultures. The inoculum was grown with continuous magnetic stirring in Pyrex Fernbach flasks (No. 4420), each containing about 1.5 l of nutrient solution. About 200 ml of the inoculum culture was used to reinoculate a fresh Fernbach flask and the remainder was used to inoculate one carboy.

The stock cultures were the only cells not dependent on nitrogen fixation, being grown on agar (1%) containing the nutrient solution given in Table I but supplemented with 20 mM KNO_3. They were kept in dim light in air as agar slants or plates prior to being transferred to liquid culture, first in cotton-stoppered test tubes (5 ml) and later in Erlenmeyer flasks (50 ml). These liquid cultures contained the inorganic nutrients given in Table I and were also supplemented with 20 mM KNO_3. After attaining a dark-blue color, two or three test tube cultures were transferred to one Erlenmeyer flask and later two to three Erlenmeyer flask cultures were used to inoculate one Fernbach flask culture. Upon transfer to the Fernbach flasks, the cultures received the nutrient solution without combined nitrogen (Table I) and became dependent on N_2 fixation as the only source of nitrogen for cellular growth.

Preparation of membrane fragments

The carboy cultures were harvested by centrifugation in a refrigerated Sharples centrifuge at a flow rate of about 1 l/min. The yield of cells grown for 4 to 5 days in the carboys was between 1 and 2 g (fresh weight) per l of nutrient solution.

The sedimented cells were gently resuspended with the aid of a tissue homogenizer in a suspending solution (twice the cell volume) containing sucrose (0.5 M),

$MgCl_2$ (10 mM), and Tricine–KOH buffer (pH 7.7) (50 mM). The suspended cells were centrifuged at $2500 \times g$ for 5 min, the supernatant was discarded, and the sedimented cells were again resuspended in (twice the cell volume) suspending solution. The cells were disrupted by passing the cell suspension under N_2 through a Ribi cell fractionator, operated between 15 000 to 18 000 lb/inch^2, at a temperature between 0 to 15 °C. About 1 mg each of DNAase and RNAase was added per about 60 ml of slurry and mixed with a tissue homogenizer. Unbroken cells and cell wall fragments were removed by centrifugation at $2500 \times g$ for 5 min and the supernatant was recentrifuged at $35\,000 \times g$ for 15 min. The pellet containing the larger photosynthetic membrane fragments (Fraction A) was resuspended in the suspending solution to give a chlorophyll concentration of 0.5 mg/ml. Fraction B, containing the smaller membrane fragments, was the pellet obtained by further centrifugation of the supernatant at $144\,000 \times g$ for 1 h.

To bring about more complete removal of phycocyanin and a possible enrichment in Photosystem I or Photosystem II, the isolated membrane fragments were subjected to a 0.5 % digitonin treatment [27]. Three parts of Fraction A were incubated with gentle stirring for 30 min at 0 °C with one part of a 2 % digitonin solution, made by dissolving digitonin in the suspending solution. The incubated mixture was centrifuged at $35\,000 \times g$ for 15 min and the pellet was discarded. The supernatant was centrifuged at $144\,000 \times g$ for 1 h and the resulting pellet was resuspended in the suspending solution (Fraction C). Each gram of fresh cells yielded Fraction A membrane fragments containing about 2 mg of chlorophyll *a*; subsequent treatment of Fraction A with digitonin yielded Fraction C membrane fragments containing about 1 mg of chlorophyll *a*.

For more complete removal of phycocyanin, Fraction C was incubated again with 0.5 % digitonin, either for an additional 30 min (Fraction D) or for 18 h (Fraction E). Chart 1 summarizes the preparation of the membrane fragments.

A slightly modified procedure was used for preparing Fraction C in assaying the effect of digitonin concentration on photochemical activity (Table IV). After incubation of Fraction A at the appropriate digitonin concentration, the supernatant from the $35\,000 \times g$ centrifugation was centrifuged at $70\,000 \times g$ for 30 min. The pellet, designated Fraction C_{70}, was collected for an activity test; the supernatant was centrifuged at $144\,000 \times g$ for 30 min, and the pellet, designated Fraction C_{144}, was also collected for an activity test.

Determination of chlorophyll and phycocyanin content

The absolute amounts of chlorophyll *a* and C-phycocyanin were determined by treating aliquots of preparations with a great excess (50 vol. or more) of 80 % acetone to extract completely chlorophyll *a* (blue-green algae do not contain chlorophyll *b* [29]). Centrifugation at $35000 \times g$ for 15 min gave an acetone solution supernatant containing chlorophyll *a* and a residue containing phycocyanin. The chlorophyll *a* in the acetone supernatant was determined spectrophotometrically at 663 nm, using the extinction coefficient of 82.04 $((mg/ml)^{-1} \cdot cm^{-1})$ [30, 31]. The residue was repeatedly extracted with 0.05 M Tricine buffer (pH 7.7) until it became colorless. The C-phycocyanin in the combined extracts was assayed spectrophotometrically at 620 nm, using the extinction coefficient of 7.3 $((mg/ml)^{-1} \cdot cm^{-1})$ of Craig and Carr [32].

Another procedure was used for a simultaneous in situ estimation of chloro-

phyll *a* and C-phycocyanin in cells and in the different membrane preparations. The chlorophyll *a* and C-phycocyanin contents of whole cells were determined on an aliquot of a sonicate made in a Branson sonicator Model S125 (power setting 4) by sonicating for 3 min whole cells suspended in 0.01 M Tricine buffer (pH 8). The chlorophyll *a* and C-phycocyanin contents of the membrane fragments were determined on suspensions of Fractions A–E obtained by the proceedure summarized in Chart 1. The extinction coefficients of chlorophyll *a* in situ were determined on a

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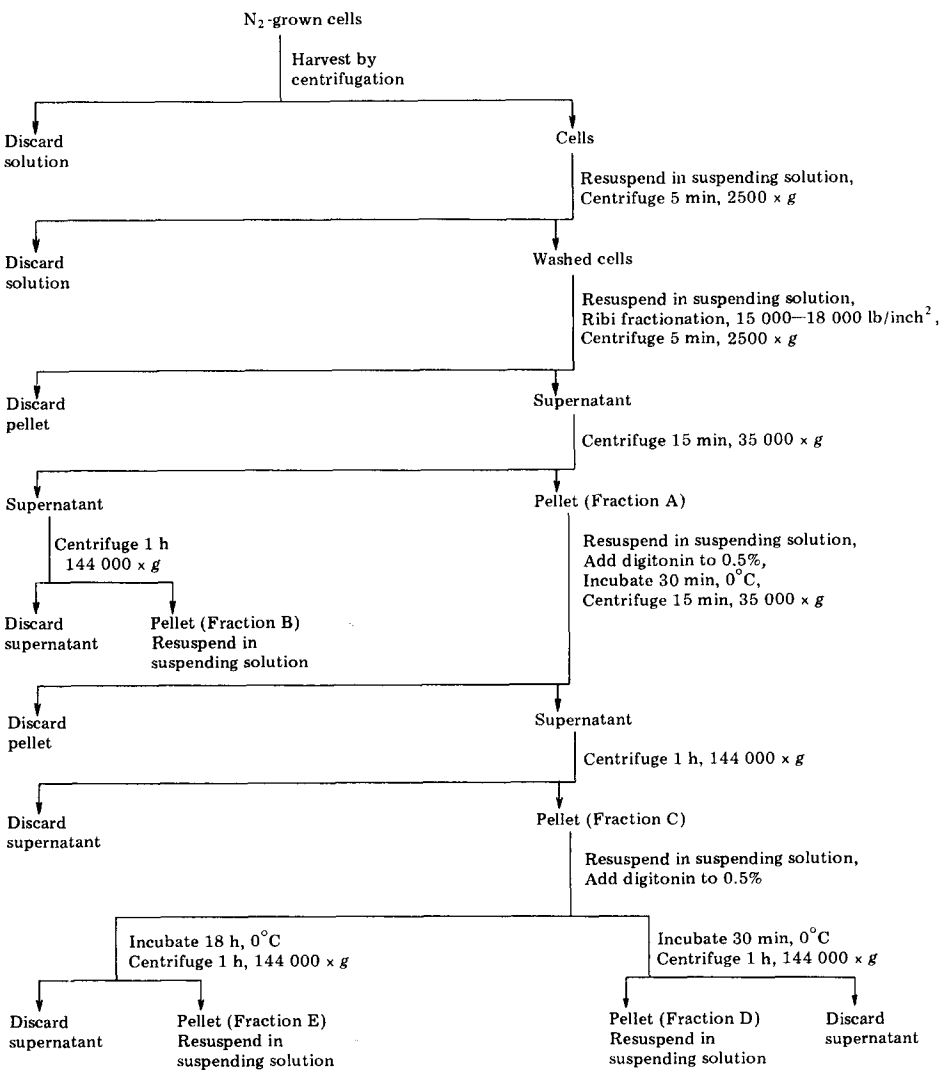

CHART 1

Fractionation of *Nostoc muscorum* cells

preparation of Fraction E that showed no remaining extractable phycocyanin. The fraction E preparation used for that purpose had an absorbance ratio $A_{620\ nm}/A_{678\ nm} = 0.528$ (see below). After determining the chlorophyll *a* content of the preparation by the 80 % acetone extraction method described, the extinction coefficients for chlorophyll *a* in situ were computed to be 67.5 $((mg/ml)^{-1} \cdot cm^{-1})$ at 678 nm (maximum chlorophyll *a* absorption in the red) and 17.3 at 620 nm.

The extinction coefficient of C-phycocyanin at 678 nm was very small; it was calculated to be 0.3 $((mg/ml)^{-1} \cdot cm^{-1})$ from the absorption spectrum of a C-phycocyanin preparation partly purified by DEAE-cellulose chromatography [33]. As already stated, the extinction coefficient used for C-phycocyanin at 620 nm was 7.3 $((mg/ml^{-})^{1} \cdot cm^{-1})$.

Using these extinction coefficients, the following two equations were set up for the simultaneous estimation of chlorophyll *a* and C-phycocyanin in the various preparations:

$$A_{678\ nm} = 67.5\ X + 0.3\ Y$$
$$A_{620\ nm} = 17.3\ X + 7.3\ Y$$

where $A_{678\ nm}$ and $A_{620\ nm}$ are the measured absorbances at 678 nm and 620 nm and X and Y are the concentrations (mg/ml) of chlorophyll *a* and C-phycocyanin, respectively.

The absorbance ratio $A_{620\ nm}/A_{678\ nm}$ provided a useful index of the completeness of the removal of phycocyanin. As already mentioned, at the lowest observed $A_{620\ nm}/A_{678\ nm}$ ratio (0.256 for Fraction E), no additional phycocyanin was solubilized by further extraction.

The progressive removal of phycocyanin from the different membrane preparations was followed spectrophotometrically by recording their absorption spectra and was expressed quantitatively by determining for each membrane fragment preparation the ratio of the two chromophores, chlorophyll *a* and phycocyanobilin. The concentration of chlorophyll *a* was computed on the premise that it exists as a single chlorophyll *a* molecule (mol. wt 893.49) attached to a protein moiety. The concentration of phycocyanobilin was computed on the basis that each C-phycocyanin monomer has a molecular weight of 36 700 and contains three phycocyanobilin chromophores with a molecular weight of 558 [34].

Absorption spectra were recorded by a Model 14 Cary spectrophotometer equipped with a scattered transmission accessory (Model 1462).

Measurements of photosynthetic activity

The photoreduction of $NADP^+$ or ferricyanide was measured by absorbance changes at 340 and 420 nm, respectively, as described by McSwain and Arnon [36]. Photophosphorylation reactions were measured by the esterification of $^{32}P_i$ and were carried out in the same cuvettes and under the same illumination conditions as used for $NADP^+$ and ferricyanide photoreduction. The [^{32}P]ATP formed was separated from the remaining $^{32}P_i$ by the method of Hagihara and Lardy [37]. Aliquots containing the labelled ATP were dried in a planchet and counted in an end-window Geiger–Müller counter.

In measuring $NADP^+$ photoreduction by *Nostoc* membrane preparations, they were supplemented with spinach ferredoxin and spinach ferredoxin–$NADP^+$

reductase, each prepared by procedures previously reported from this laboratory (see ref. 38 and 39). Digitonin was purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Changes in chlorophyll to phycocyanin ratio

The progressive loss of phycocyanin from the different membrane preparations is illustrated by Fig. 1. Here, the absorption spectra (in the region 550–700 nm) of the membrane preparations are compared with those of whole cells containing the same amount of chlorophyll *a*, based on equal absorbance at 678 nm. The loss of phycocyanin is indicated by the progressive decreases in absorbance at 620 nm.

The two major steps in the removal of phycocyanin were (i) the disruption of the cells in an aqueous medium (compare the 620-nm absorbance peaks of Fractions A and B with that of whole cells) and (ii) the subsequent digitonin treatment.

A short (30 min) digitonin (0.5 %) treatment converted Fraction A into Fraction C, whose much smaller absorbance in the 620-nm region indicated a sharp additional decrease in phycocyanin content (Fig. 1). A subsequent 30-min treatment of Fraction C with 0.5 % digitonin resulted in a further loss of phycocyanin (Fraction

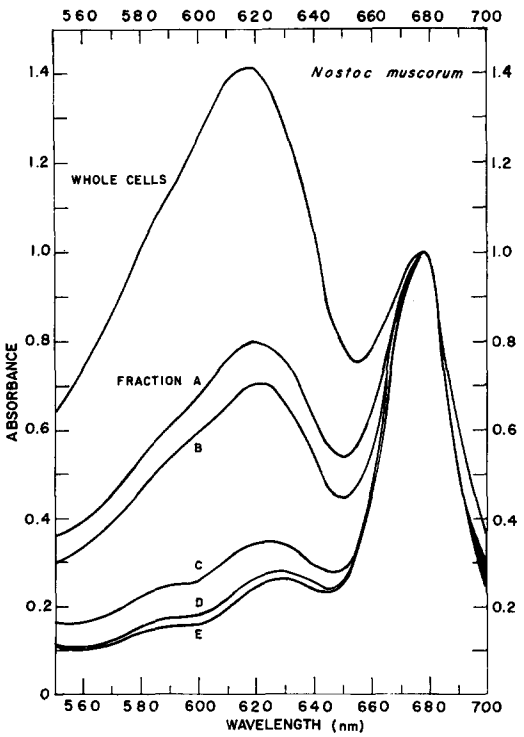

Fig. 1. Phycocyanin content of whole cells and membrane fragments containing equal amounts of chlorophyll *a* as reflected in absorbance changes in the 620-nm region. (For experimental details see Methods).

D). The most complete removal of phycocyanin from the membrane fragments was obtained by treating Fraction C with 0.5 % digitonin for 18 h (Fraction E). However, the amount of phycocyanin removed by the long digitonin treatment was small relative to the phycocyanin removed by the initial 30-min digitonin treatment (Fraction C vs Fraction A).

The drastic alteration in pigment composition of Fraction E, relative to that of whole cells, is shown by a comparison (based on equal chlorophyll *a* absorption at 678 nm) of the respective absorption spectra extended over the range from 400 to 720 nm (Fig. 2). As indicated by the markedly lower absorbance at wavelengths between about 450 and 670 nm, the membrane fragments in Fraction E lost not only phycocyanin but also carotenoids, flavins, and other constituents that will be left to future investigations.

Since one long-range objective in starting this investigation was to compare the photosynthetic apparatus and mechanisms in membrane fragments from blue-green algae with those in chloroplasts, it was deemed useful to compare at the start the absorption spectra of Fraction E and of washed spinach chloroplasts (P_{1S} [35] that had equal absorbance at 678 nm. As expected, the major difference between the two spectra was the diminished absorbance by Fraction E in the regions around 460 and 650 nm caused by the absence of chlorophyll *b* (Fig. 3). There were other differences between the two absorption spectra but the evaluation of these is deferred to future investigations.

The relative abundance of the two chromophores, chlorophyll *a* and phycocyanobilin, in the different membrane preparations and whole cells is summarized in Table II. The molar ratio of chlorophyll *a* to phycocyanobilin was about 370 times greater in Fraction E than in whole cells; some preparations of Fraction E had no measurable phycocyanin at all.

Photosynthetic activity of membrane fragments

Typical photosynthetic activity of the different membrane fragments prepared from the same batch of cells is summarized in Table III. A striking and unexpected feature of all of the membrane preparations (Fractions A–E) was their capacity for the photoreduction of $NADP^+$ by water (indicative of Photosystem II activity) which remained undiminished despite the total or nearly total removal of phycocyanin by

TABLE II

CHLOROPHYLL *a* AND PHYCOYANOBILIN IN WHOLE CELLS AND MEMBRANE FRAGMENTS OF *Nostoc muscorum* (Strain 7119)

Preparation	Chlorophyll *a*★ (nmoles/ml)	Phycocyanobilin★ (nmoles/ml)	Molar ratio Chlorophyll *a* to Phycocyanobilin
Whole cells	6.16	4.65	1.32
Fraction A	11.76	4.65	2.54
Fraction B	12.21	3.90	3.13
Fraction C	9.30	0.410	22.7
Fraction D	14.45	0.273	52.9
Fraction E	102.48	0.210	488.0

★ For basis of estimation, see Methods.

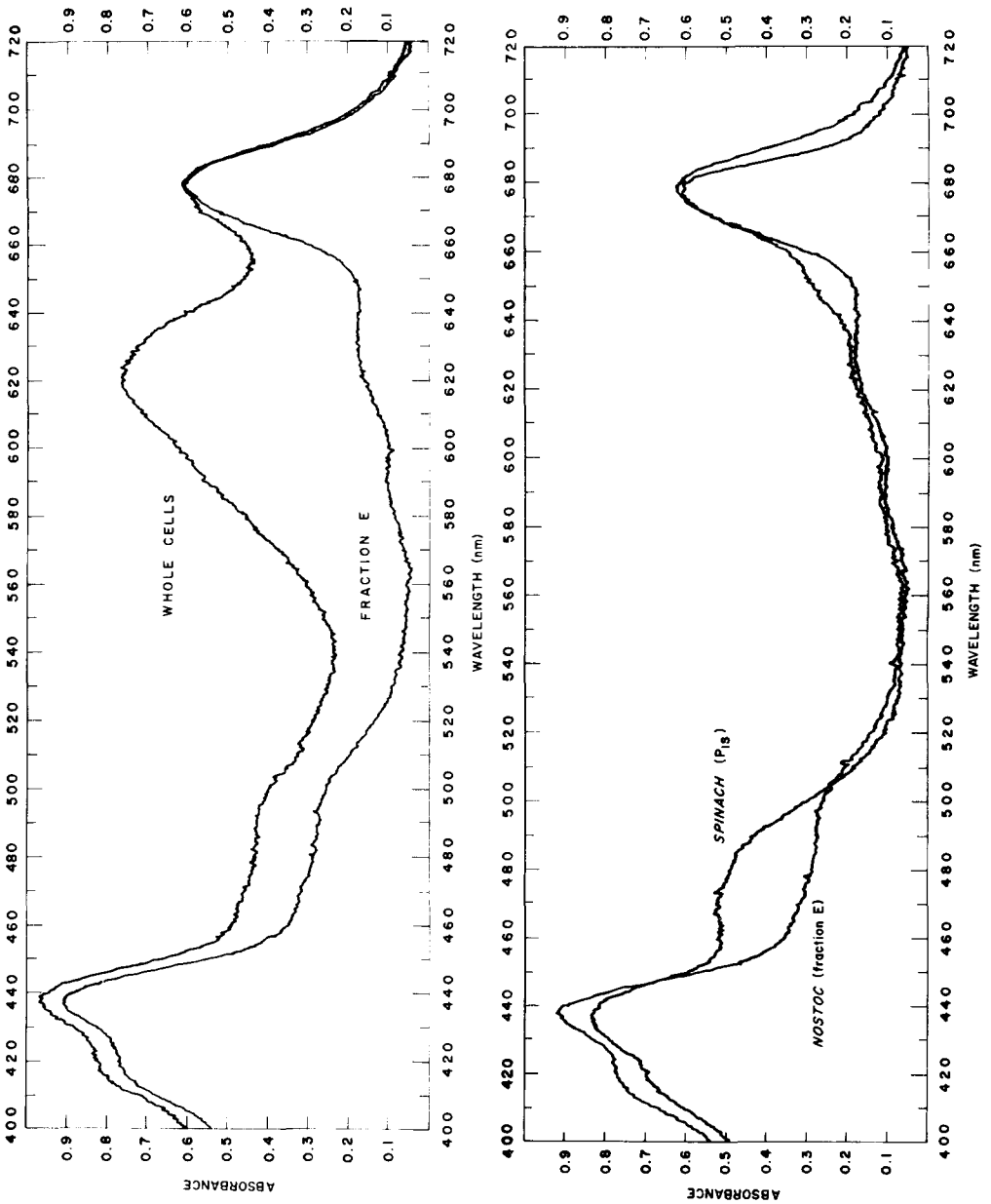

Fig. 2. Absorption spectra of whole cells and membrane fragments (Fraction E) based on equal chlorophyll *a* content.

Fig. 3. A comparison of the absorption spectra of *Nostoc* membrane fragments (Fraction E) and spinach chloroplasts based on equal absorption at 678 nm.

TABLE III

PHOTOSYNTHETIC ACTIVITY OF MEMBRANE PREPARATIONS FROM *NOSTOC MUSCORUM* (Strain 7119)

The reaction mixture contained (per 1.0 ml) membrane fragments (equivalent to 50 μg chlorophyll *a*) and the following: 50 μmoles Tricine (*N*-tris(hydroxymethyl)methylglycine) buffer (pH 7.7), 10 μmoles $MgCl_2$; 2 μmoles ADP and 2 μmoles $K_2H^{32}PO_4$. Other additions were: (i) to the ferricyanide system, 4 μmoles $K_3Fe(CN)_6$; (ii) to the $H_2O \rightarrow NADP^+$ system, 0.1 μmole ferredoxin, 2 μmoles $NADP^+$ and saturating amounts of ferredoxin–$NADP^+$ reductase; (iii) to the $DCIPH_2 \rightarrow NADP^+$ system, the same additions as under (ii) above plus 10 μmoles ascorbate, 0.1 μmole DCIP and 0.001 μmole DCMU; and (iv) to the cyclic system, 0.05 μmole phenazine methosulfate. Gas phase, air; temperature, 20 °C; time, 3 min. Illumination ($2.6 \cdot 10^5$ ergs/cm$^2 \cdot$ s^{-1}) was provided by a light beam from a 1200-W projection lamp filtered through wide-band red filters (Corning 2–64 and 1–69). Q_{2e} = μmoles $NADP^+$ or 0.5 μmole ferricyanide reduced/mg chlorophyll/h. Q_{ATP} = μmoles ATP formed/mg chlorophyll/h.

Membrane preparation	$H_2O \rightarrow$ ferricyanide		$H_2O \rightarrow NADP^+$		$DCIPH_2 \rightarrow NADP^+$		Cyclic photophosphorylation
	Q_{2e}	Q_{ATP}	Q_{2e}	Q_{ATP}	Q_{2e}	Q_{ATP}	Q_{ATP}
Fraction A	196	72	177	85	84	36	164
Fraction B	156	64	142	89	126	44	129
Fraction C	124	11	148	12	238	19	49
Fraction D	176	6	158	10	293	17	42
Fraction E	140	2	122	1	222	6	18

successive digitonin treatments, one of which lasted 18 h (Fraction E). The rates of $NADP^+$ photoreduction by water for the various membrane preparations (including Fraction E) were often as high as 300 μmoles of NADPH formed per mg of chlorophyll per h. Similar rates for $NADP^+$ reduction by Photosystem I alone (reduced 2,6-dichlorophenolindophenol ($DCIPH_2$) $\rightarrow$ $NADP^+$) were also obtained with membrane fragments treated with digitonin (Fractions C, D and E).

The rates of light-induced electron transport from water to $NADP^+$ or from $DCIPH_2$ to $NADP^+$ could be preserved in the different membrane preparations for at least a month (and probably for considerably longer periods of time) by freezing and storing them at −20 °C.

In Fractions A and B, which were not treated with digitonin, the rates of non-cyclic photophosphorylation (Q_{ATP}) were approximately one-half of the rates (Q_{2e}) of the coupled electron transport from water to $NADP^+$ or ferricyanide. Fractions A and B also had appreciable cyclic photophosphorylation activity (catalyzed by phenazine methosulfate) and photophosphorylation linked to $NADP^+$ reduction by $DCIPH_2$. All photophosphorylation activity was sharply decreased by the digitonin treatments (Fractions C and D) and was practically abolished by the 18-h digitonin treatment (Fraction E).

Effect of digitonin concentration on light-induced electron transport

Since all *Nostoc* membrane fragments treated with 0.5 % digitonin continued to exhibit substantial activity of both Photosystems I and II (as indicated by their photoreduction of $NADP^+$ with water or $DCIPH_2$ (Table III) we treated the *Nostoc* membranes with higher concentrations of digitonin and measured its effect on the photoreduction of $NADP^+$). Table IV shows that digitonin at concentrations as

high as 2 or 4 % failed to bring about separation of the photosystems. Photosystem I activity ($DCIPH_2 \rightarrow NADP^+$) of the membrane fragments was usually increased by the digitonin treatment, both in the larger fragments sedimented at $70\,000 \times g$ (Fraction C_{70}) and in the smaller fragments sedimented at $144\,000 \times g$ (Fraction C_{144}), but all membrane fragments retained the capacity to photoreduce $NADP^+$ with water at relatively high rates.

TABLE IV

EFFECT OF DIGITONIN CONCENTRATION ON PHOTOREDUCTION OF $NADP^+$ BY MEMBRANE FRAGMENTS FROM *NOSTOC MUSCORUM* (Strain 7119)

Experimental conditions as described in Table III except that $K_2H^{32}PO_4$ was replaced by K_2HPO_4 and time was 1 min.

Membrane fragments	Digitonin (%)	Photoreduction of $NADP^+$ (μmoles/mg chlorophyll/h)	
		$H_2O \rightarrow NADP^+$	$DPIPH_2 \rightarrow NADP^+$
Fraction A	0	142	305
Fraction C_{70}	0.5	175	305
Fraction C_{144}	0.5	175	330
Fraction A	0	245	136
Fraction C_{70}	2.0	169	262
Fraction C_{144}	2.0	114	212
Fraction A	0	250	175
Fraction C_{70}	4.0	165	262
Fraction C_{144}	4.0	195	340

Action spectra and quantum efficiency

Other evidence that membrane fragments substantially freed of phycocyanin (Fraction C) were able to use variants of a single pigment, chlorophyll *a*, to operate both Photosystems I and II was sought from action spectra and relative quantum efficiency measurements of $NADP^+$ photoreduction by water and by $DCIPH_2$.

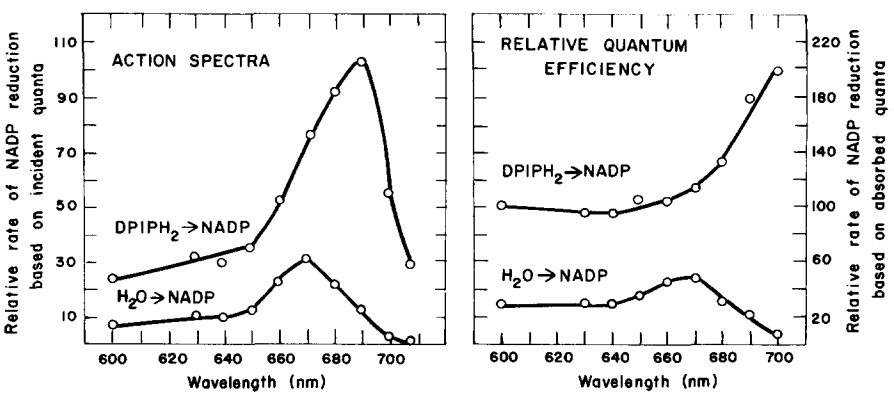

Fig. 4. A comparison of action spectra and relative quantum efficiency of $NADP^+$ photoreduction with water and reduced DCIP by *Nostoc* membrane fragments (Fraction C). The reaction mixture was as described for Table IV. The experimental procedure is given in [36].

Fig. 4 shows that the action spectra of the two kinds of light-induced electron transport were different. The action spectrum of the photoreduction of $NADP^+$ by water had a maximum at about 670 nm, whereas the action spectrum of the photoreduction of $NADP^+$ by $DCIPH_2$ had a maximum at about 690 nm. The difference between the two systems is also reflected in their contrasting utilization of far-red monochromatic light. Based on absorbed quanta, the relative quantum efficiency of NADP photoreduction by water showed a decline or a "red drop" at wavelengths longer than 670 nm with the reaction coming essentially to a halt at wavelengths longer than 700 nm. By contrast, the photoreduction of $NADP^+$ by $DCIPH_2$ showed a "red rise" [25], with the relative quantum efficiency rising at wavelengths longer than 690 nm (Fig. 4).

DISCUSSION

The removal of phycocyanin from *Nostoc* membrane fragments altered drastically their natural pigment relationships, as reflected in an almost 400-fold increase in the ratio of chlorophyll to phycocyanin in some preparations. Nevertheless, all membrane preparations, including those from which no more phycocyanin could be extracted by the methods used here, retained the capacity for the photoreduction of NADP or ferricyanide by water. Thus, despite its great importance in whole cells as the main light-gathering pigment, phycocyanin appears not to be essential for photosynthetic electron transport in *Nostoc* membrane fragments. In these, chlorophyll *a*, in association with other membrane components, is responsible for light-induced electron transport.

Since chlorophyll *a* is the only kind of chlorophyll present in blue-green algae, their membrane fragments depleted of phycocyanin provide especially favorable material for demonstrating the presence of different chlorophyll *a* photosystems [40]. The coexistence in *Nostoc* membrane fragments of two different chlorophyll *a* photosystems was evidenced by their different action spectra and by their contrasting efficiency in the utilization of far-red quanta in light-induced electron transport (Fig. 4). The membrane fragments used in these experiments (Fraction C), though depleted of phycocyanin (Fig. 1), still retained a measurable amount of this pigment (Table II) but its presence did not seem to influence either the action spectra or the relative quantum efficiency of electron transport at the peak of phycocyanin absorbance (620 nm). These findings reinforce the conclusion that the functional integrity of photosynthetic electron transport in *Nostoc* membranes does not depend on phycocyanin but is tied directly to the functioning of different chlorophyll *a* photosystems.

Turning to photophosphorylation, cyclic photophosphorylation appears to be independent of phycocyanin (see Introduction) but its need for noncyclic photophosphorylation is still not settled because the mild cell disruption methods used by earlier investigators [12–15] to demonstrate noncyclic photophosphorylation probably left appreciable amounts of phycocyanin attached to the cell fragments. No final conclusions can be drawn from this study about a possible role of phycocyanin in either cyclic or noncyclic photophosphorylation. Our drastic digitonin treatments for phycocyanin removal abolished all types of photophosphorylation but the explanation of this effect is reserved for future study.

It should be noted that phycocyanin may have some hitherto unrecognized

photochemical function(s) in cells. For instance, Pjon et al. [41] isolated from *A. cylindrica* a photochemically active chromoprotein (whose physiological role is unknown) that appears to be a complex of C-phycocyanin and a pigment with an absorption maximum at 695 nm.

The adequacy of chlorophyll *a* photosystems for supporting a complete light-induced electron flow from water to $NADP^+$ in *Nostoc* membrane fragments from which phycocyanin has been removed is in accord with the findings of Lemasson et al. [2] that chlorophyll *a* can be made to serve as the major light-harvesting pigment in intact cells in which the formation of phycobiliproteins was experimentally diminished by nitrogen deprivation. Our results are also in general agreement with the work of Volk and Bishop [42] who found that photosynthesis and Hill reaction were not impaired in a phycocyanin-free mutant of *Cyanidium caldarum* (an alga which, despite having phycocyanin in the wild type, is a eucaryote included among the *Rhodophyta* rather than among blue-green algae [43]).

An unexpected finding was the failure of even drastic digitonin treatments to bring about a separation of photosystems in the *Nostoc* fragments. These results invite attention to those of Anderson and Vernon [44] who found the digitonin treatment ineffective for separation of Photosystems I and II in spinach chloroplasts isolated in low-salt Tricine buffer. Such chloroplasts exhibited "a general breakdown of grana structure to a less well-defined membrane system" resembling in part the findings of Izawa and Good [45]. Our experience with *Nostoc* membranes, which do not form grana, accords with the expectation of Anderson and Vernon [44] that digitonin may not be able to release Photosystem I particles from chloroplasts devoid of grana. Membrane fragments and reaction centers from blue-green algae have been described by other investigators, e.g. Ogawa and Vernon [46], Suzuki and Fujita [47], and Dietrich and Thornber [48], whose objectives and methods were different from ours. The characteristic feature of all of our membrane preparations was the stability of their photosynthetic electron transport from water to $NADP^+$. Whether this stability was due to the organism selected or to the methods of cell culture and membrane fragmentation has not been determined.

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