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ELECTRON TRANSFER COMPONENTS OF WILD-TYPE AND PHOTOSYNTHETIC MUTANT STRAINS OF *SCENEDESMUS OBLIQUUS* D_3

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SUMMARY

To investigate the possible alteration of various components of the photosynthetic electron transport system of certain mutants of Scenedesmus techniques were developed for their extraction and purification from whole cells of this alga. The components identified in the normal alga were cytochrome *c* 549, cytochrome *b* 562, a cytochrome *c* 551, flavoprotein–ferredoxin reductase, plastocyanin, cytochrome *c* 552, and ferredoxin. Lamellar-bound cytochrome *c* 552 and cytochromes *b* were also detected. Application of the extraction and purification techniques to two photosynthetic mutants revealed that Mutants 26 and 50 lacked cytochrome *f* in both the bound and soluble forms (Mutant 50) or in only the bound form (Mutant 26). Chloroplasts prepared from either of these mutants lacked Hill reaction activity with a variety of oxidants with water as the electron donor but photoreduced $NADP^+$ with 2,6-dichlorophenolindophenol and ascorbate as the electron donor system. No photophosphorylation *in vivo* was detected with either mutant, but isolated chloroplasts performed a cyclic photophosphorylation with phenazine methosulphate as cofactor. Fluorescence analysis revealed that both mutants possess a measurable Photosystem II activity.

It was concluded that the loss of cytochrome *f* prevents the normal flow of electrons from Photosystem II to NADP and also to a variety of other Hill reaction oxidants. Furthermore, cytochrome *f* is not required for the reduction of NADP with electron donor systems other than water nor is it an essential component of the mechanism of cyclic photophosphorylation with phenazine methosulphate as cofactor.

INTRODUCTION

The use of selectively produced photosynthetic mutants of unicellular algae is now regarded as an indispensable tool for gaining further insight into the mechanism of the photosynthetic processes. This approach has been successfully employed by LEVINE[1] with Chlamydomonas and by BISHOP[2] with Scenedesmus.

Subdivision of these photosynthetic mutants into classes based upon their

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PMS, phenazine methosulphate.

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ability to perform the various partial reactions of photosynthesis has been possible. BISHOP[2] originally classified the photosynthetic mutants of Scenedesmus as either oxygen mutants which lack *p*-benzoquinone Hill reaction activity but perform photoreduction of CO_2 with H_2, or carbon dioxide mutants which show no photoreduction *in vivo* but perform a normal *p*-benzoquinone Hill reaction. More recent interpretations of the mechanism of photosynthesis suggest that such mutants should be classified as Photosystem II and Photosystem I mutants, respectively.

More recently PRATT AND BISHOP[3] described two photosynthetic mutants of Scenedesmus which do not fit such a classification. These mutants, 26 and 50, are typical oxygen mutants in many respects; the photosynthetic particles isolated from them lack Hill reaction activity but will photoreduce NADP with the 2,6-dichlorophenolindophenol (DCIP)–ascorbate couple. Furthermore, such particles carry out phenazine methosulphate (PMS)-mediated cyclic photophosphorylation.

However, whole cells of these mutants lack both photoreduction with hydrogen and cyclic photophosphorylation as measured by anaerobic glucose assimilation. The reactions mentioned require an active Photosystem I which is apparent in isolated chloroplasts but not in whole cells of these mutants. Consequently, it was considered that the defects of these two mutants must be in the photosynthetic electron transfer system connecting the two photosystems. Mutants 50 and 26 have normal concentrations of plastoquinone, ferredoxin, hydrogenase and P-700 (from electron paramagnetic resonance studies); hence, it is possible that the defects involve either a cytochrome (soluble or membrane bound), or plastocyanin.

This paper describes the procedure for the extraction and partial purification of both the soluble and membrane-bound electron transport components of whole Scenedesmus cells. Also included are the comparative studies made on Mutants 26 and 50 in regard to the relationship between photosynthetic activity and the presence or absence of certain components of the photosynthetic electron transport system.

METHODS AND MATERIALS

The procedure for the culture of the algae and for the isolation of photosynthetic particles has been previously described[3].

Substituted celluloses, CM-32 and DE-32, were obtained from Whatman.

Pyridine hemochromogens were prepared as described by VERNON AND KAMEN[4].

Hemes were cleaved from cytochromes with acid–acetone and the acid non-dissociable hemes were extracted according to PAUL[5].

The oxidation–reduction potentials of various cytochromes were determined as described by DAVENPORT AND HILL[6].

Preparation of crude protein extract

In a typical experiment 118 g fresh weight of packed cells of wild-type *Scenedesmus obliquus* were resuspended in 0.2 M Tris–HCl, pH 7.5, to which had been added a small amount of deoxyribonuclease to degrade the DNA released on cell breakage. The algal cells were broken twice in the French press at 24000 lb/inch^2. The broken cell suspension was made 2 % with respect to Triton X-100 and incubated overnight at 2°. After removal of the cell debris by centrifugation (25000 × *g* for 15 min) the dark-green supernatant was brought to 35 % saturation with solid $(NH_4)_2SO_4$. After

standing for 10 min the suspension was centrifuged at 25000 × *g* for 30 min. The yellow-brown solution was separated from the floating green lipoprotein material by decantation and by filtration through glass wool. The yellow-brown solution was brought to 90 % saturation with solid $(NH_4)_2SO_4$ and allowed to stand for 1 h at 2° and then centrifuged. An outline of this procedure is shown in Fig. 1. The reddish-brown pellet so obtained was suspended in distilled water and dialyzed overnight to remove $(NH_4)_2SO_4$.

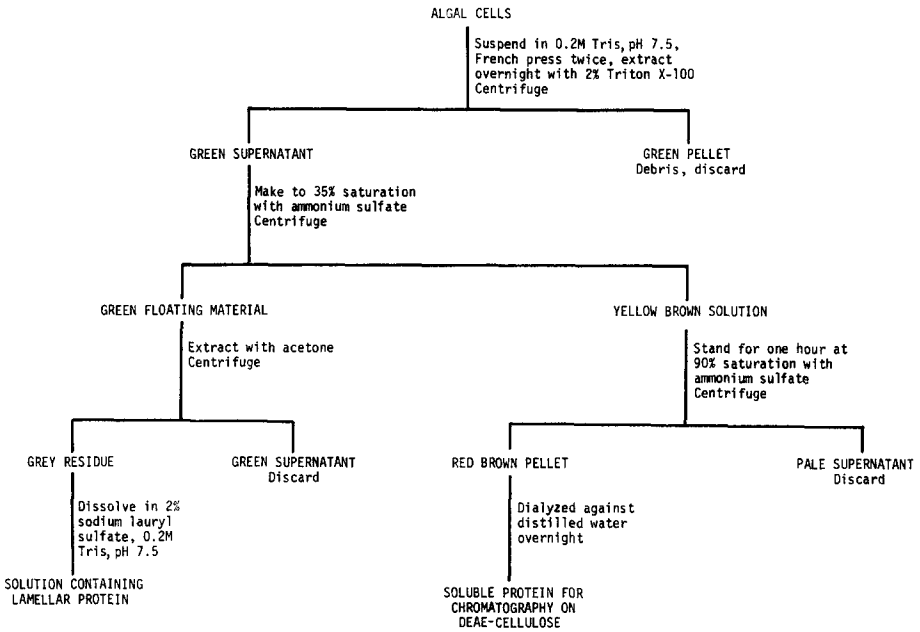

Fig. 1. General scheme for the isolation of soluble proteins from whole cells of *Scenedesmus obliquus*.

The green floating material was exhaustively extracted with acetone (—15°), until free of chlorophyll. The gray powder so obtained was freed of acetone and dissolved in 3 % sodium dodecyl sulfate in 0.2 M Tris–HCl (pH 7.5). The resulting green-brown solution served as a crude solution of the lamellar protein.

RESULTS AND DISCUSSION

The dialyzed extract was adsorbed onto a 15 cm × 2 cm column of DEAE-cellulose (Whatman DE-32, microgranular) and the adsorbed protein was eluted with an increasing gradient of Tris–HCl (pH 7.5). A mixing chamber contained 500 ml of distilled water and the reservoir 1 l of 1.0 M Tris–HCl (pH 7.5). Fractions of 5 ml were collected commencing with the application of the extract to the column. Details of the chromatography are shown in Fig. 2.

A pink fraction (Tubes 0–4) was not adsorbed to the column; its absorption spectrum indicated that it was reduced cytochrome *c* 549. Other proteins of interest which were adsorbed to the column and progressively eluted were cytochrome *b* 562, reduced cytochrome *c* 551, the flavin enzyme $NADP^+$–ferredoxin reductase, cyto-

chrome *b* 558, reduced plastocyanin, reduced cytochrome *c* 552 and ferredoxin (Table I). Apart from cytochrome *c* 549, these proteins were purified by repeated chromatography on narrow columns of DEAE-cellulose, followed by gel filtration on Sephadex G-200. This enabled all the components to be completely separated from one another. Cytochrome *c* 549 was purified by gradient elution from CM-cellulose followed by gel filtration on Sephadex G-200.

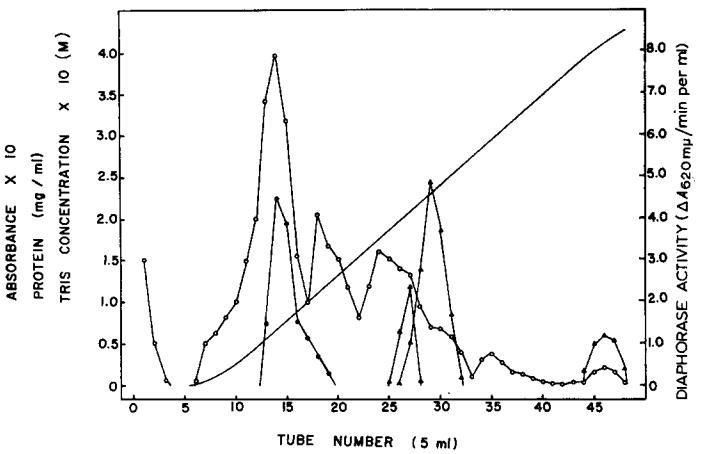

Fig. 2. Elution pattern obtained from the DEAE-32 cellulose chromatography of the 35–90% saturated $(NH_4)_2SO_4$ fraction of a 2% Triton X-100–0.2 M Tris, pH 7.5, extract of 118 g (wet weight) of Scenedesmus. ———, Tris–HCl gradient; O—O, protein concentration (mg/ml); ▲—▲, absorbance of plastocyanin (Tubes 25–28), of cytochrome *f* (Tubes 26–32), and ferredoxin (Tubes 44–48). ●—●, diaphorase activity ($\Delta A_{620\ nm}$/min per ml).

TABLE I

ABSORPTION CHARACTERISTICS OF SOLUBLE CYTOCHROMES EXTRACTED FROM SCENEDESMUS

Cytochrome	*γ*	*β*	*α*	*Oxidized γ*	*Ratio γ/α*	*Remarks*
c 549	417	521	549	412	4.65	Basic protein
b 562	433	532	562	419	4.92	Reducible with ascorbate. Large increase in Soret absorption on reduction
c 551	422	522	551		7.1	Symmetrical α peak
b 558	426	527	558	413		Autoxidizable, not reduced by ascorbate
c 552 (*f*)	417	521	552	412	6.9	Unsymmetrical α peak

Cytochrome c 549

This basic protein was eluted almost entirely in the reduced form after adsorption to CM-cellulose and showed absorption maxima at 417, 521 and 549 nm; on oxidation the Sorêt peak shifted to 412 nm. Reduction occurred with ascorbate as well as dithionite. The reduced α peak was completely symmetrical at pH 7.0, and the absorbance ratio $A_{471\ nm}/A_{549\ nm}$ was 4.6. After splitting the heme from the

protein with Ag_2SO_4, the pyridine hemochromogen showed absorption characteristics of a mesoheme prosthetic group.

Cytochrome *c* 549 is probably of mitochondrial origin in view of its marked similarity to the mitochondrial cytochrome *c* isolated from plants and animals. This similarity is particularly well shown by its basicity and its low γ/α absorbance ratio. HOLTON AND MYERS[18] have isolated a cytochrome *c* 549 from *Anacystis nidulans* whose γ/α absorbance ratio was 7.5. Because of the agreement of this value with that of algal cytochrome *c* 552, they concluded that the 549 species of Anacystis was related to the photosynthetic *c* (or *f*-type) cytochrome and not to the mammalian cytochrome *c* of the respiratory chain.

Cytochrome b 562

Cytochrome *b* 562 eluted from DEAE-cellulose (Tubes 10–14) in the oxidized form and showed Sorêt absorption at 419 nm. On reduction with either ascorbate or dithionite selective absorption occurred at 433, 532 and 562 nm. The reduced protein was slightly autoxidizable. Cleavage with acid–acetone led to the identification of the prosthetic group as protoheme.

This cytochrome closely resembles two other soluble cytochromes *b* described in the literature, Monostroma cytochrome *b* 562 (ref. 7) and mung bean seedling (*Phaeseolus aureus*) cytochrome *b* 561 (ref. 8). The intracellular location of these cytochromes is not known definitely; the mung bean cytochrome is possibly a microsomal component. However, there remains a possibility that the Scenedesmus cytochrome *b* 562 is a soluble chloroplast component, although so far its absence has not been detected in a photosynthetic mutant, nor has it been examined for its reactivity with algal chloroplast preparations.

Cytochrome c 551

This cytochrome, always isolated in the reduced form (Tubes 13–16), has absorption maxima at 422, 522 and 551 nm. The γ/α absorption ratio was 7.1 and the α peak was completely symmetrical. The function of this cytochrome is unknown. Preliminary observations indicate that it is in some way related to cytochrome *f*, since the concentration of cytochrome *c* 551 is higher than normal in some of the photosynthetic mutants which have decreased levels of cytochrome *f*. Possibly such a relationship is borne out by its γ/α ratio which is the same as the cytochrome *f* value and larger than other cytochromes. It must also be considered that this component represents a modified form of cytochrome *c* 552 due to the extraction and purification procedure.

Flavoprotein, ferredoxin–NADP$^+$ reductase

This enzyme was eluted from DEAE-cellulose along with cytochrome *c* 551 (Tubes 13–17). However, these two components could be separated by filtration on Sephadex G-200. The enzyme was estimated in fractions eluted from the column by means of its NADP$^+$ diaphorase activity[9].

Cytochrome b 558

This cytochrome was always isolated in the oxidized form (Tubes 22–25) and possessed a Sorêt absorption maximum at 413 nm. It was not reducible with ascorbate,

and on reduction with dithionite absorption maxima of 426, 527 and 558 nm were exhibited. This cytochrome is similar to cytochrome b_3 isolated from broad bean leaves[10] and from etiolated mung been seedlings[11] and is considered to be of microsomal origin. However, the Scenedesmus cytochrome differs in its ease of reduction with ascorbate and its stability towards acetone. Recently a similar cytochrome of chloroplast, cytochrome *b* 559, has been reported[12]. This cytochrome is reducible with ascorbate and is sensitive to acetone[13]. This component, in contrast to cytochrome b_3, is firmly bound to the chloroplast and has not been isolated to date. The cellular localization of Scenedesmus cytochrome *b* 558 remains unknown as it has not been possible to fractionate the cellular components of Scenedesmus. However, this cytochrome, as isolated from whole cells of Scenedesmus, is probably not of chloroplast origin.

Plastocyanin

Plastocyanin was eluted from the column (Tubes 26–28) in the reduced form; addition of ferricyanide to individual tubes localized this component by its blue color and 598-nm absorbance maximum. Its properties were similar to plastocyanin isolated from other higher plants and algae[14,15].

Cytochrome c 552

Cytochrome *c* 552 was isolated in the reduced form (Tubes 28–31) indicating its lack of autoxidizability. Absorbance maxima were shown at 417, 521 and 552 nm; on oxidation the Sorêt absorption peak was 412 nm. This cytochrome has all the properties now considered to be typical of algal cytochrome *f* (refs. 16–20). This type of cytochrome, which has now been isolated and purified from a considerable number of algae, has an asymmetric α peak, an E'_0 about +0.35 V and a γ/α absorbance ratio of approx. 7. Scenedesmus cytochrome *c* 552 has an asymmetric α peak, an E'_0 of +0.33 V and a γ/α absorbance ratio of 6.92.

Ferredoxin

Ferredoxin was eluted from DEAE-cellulose column (Tubes 44–48) as a marked brown-red band. The oxidized protein absorbed light at 330, 421 and 464 nm. Its properties were similar to those described by MATSURBA[21].

A summary of the general characteristics of the various electron transport components discussed above is presented in Table I.

Membrane bound components

Ferricyanide difference spectra of the solubilized lamellar protein (see METHODS AND MATERIALS) indicated the presence of a reduced cytochrome absorbing at 552 nm. The asymmetry of the α absorbance peak suggested that it was cytochrome *f*. This cytochrome has previously been shown to occur in photosynthetic organisms in two forms, one easily solubilized and the other tightly bound[22].

A dithionite difference spectrum also revealed the presence of *b*-type cytochromes. A broad absorption peak was obtained which centered at 558 nm; this is probably due to the combined absorption of cytochrome b_6 (reported α peak 562 nm (ref. 10)) and chloroplast cytochrome *b* 559. The latter has been reported to be tightly bound to the chloroplast and normally reducible with ascorbate; however, after

acetone treatment this cytochrome is only reducible with dithionite[13]. No reduction of these two cytochrome components was obtained with ascorbate. In the presence of dithionite a marked decrease in absorption occurred also in the region of 492 nm. This may possibly be due to the reduction of membrane-bound rubimedin[23] or to a reduced form of the remaining chlorophyll. No similar absorption change occurred in the presence of ascorbate.

Properties of mutants

When photosynthetic Mutants 50 and 60 were examined by the previously outlined procedures it was possible to identify some of their biochemical lesions. Protein extracted from Mutant 50 resembled that of the wild-type except for a lack of cytochrome *f* in the eluent from the DEAE-cellulose column (Fig. 3). Moreover, the lamellar protein was also deficient in cytochrome *f* as shown by the ferricyanide difference spectrum (insert of Fig. 3). The soluble proteins isolated from Mutant 26 were identical to those of the wild type. However, the dithionite difference spectrum of the lamellar protein fraction indicated the absence of cytochrome *b*. Surprisingly, the ferricyanide difference spectrum also showed a lack of lamellar cytochrome *f*, despite the presence of this cytochrome in the solubilized form (Fig. 4). A possible explanation for this property is that the mutation has led to the loss of a component which is responsible for binding the cytochromes to the lamellar membranes.

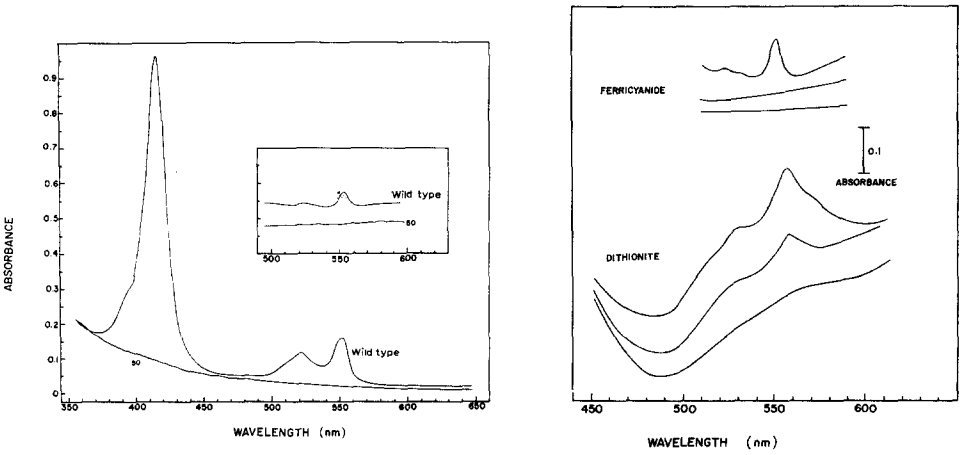

Fig. 3. Absorption spectra of the fractions from the DEAE-cellulose column normally containing cytochrome 552. Note the absence of appreciable absorbance in fractions obtained from Mutant 50 as caused by cytochrome 552. Insert: Oxidized–reduced difference spectra of the detergent solubilized lamellar proteins of wild type and Mutant 50. Oxidation was carried out with either $K_3[Fe(CN)_6]$ or NaClO.

Fig. 4. Absorption spectra of oxidized or reduced lamellar protein extracts of wild type (upper curve), Mutant 50 (middle curve) and Mutant 26 (lower curve).

Examination of the fine structure of the chloroplast of the two mutants has shown that in Mutant 26 a decrease in the number of lamellae has occurred and furthermore that most of the remaining lamellae have fused into amorphous units. The structure of the chloroplasts of Mutant 50 were identical to that of the wild-type chloroplasts. Details of the chloroplast ultrastructure will be published elsewhere.

TABLE II

PHOTOCHEMICAL ACTIVITIES OF CHLOROPLASTS OF SCENEDESMUS

Rates are expressed as a percentage of control (wild-type cells).

Chloroplast source	*DCIP**	*Cytochrome c**	*$NADP^+$**	*DCIP–ascorbate** $NADP^+$*	*PMS-catalyzed photophosphorylation*
Mutant 26	2	2	10	52	28
Mutant 50	2	2	10	45	72
Mutant 11	0	2	10	77	100
Mutant 8	36	15	10	16	0

* Hill reaction rates with water as the electrons donor. The rates for the individual reactions of wild-type chloroplasts (control) were as follows: DCIP, 75; cytochrome *c*, 130; $NADP^+$, 96; and $NADP^+$ (DCIP–ascorbate), 74. All rates expressed as μmoles oxidant reduced/mg chlorophyll per h. The control rate of photophosphorylation was 25 μmoles ATP esterified/mg chlorophyll per h. For details of methods see ref. 3.

** DCMU ($5 \cdot 10^{-6}$ M) added to prevent contributions from Photosystem II.

The lack of detectable cytochrome *f* in Mutant 50, in either the soluble or bound form, is of interest in that photochemical active fragments isolated from this mutant strain carry out PMS-mediated cyclic photophosphorylation as efficiently as do preparations from wild type (Table II). Thus it appears that cytochrome *f* is not involved in PMS-mediated cyclic photophosphorylation of Scenedesmus chloroplasts; this finding supports earlier observations by LEVINE[1] for Chlamydomonas mutants. However, cyclic photophosphorylation *in vivo*, as measured by anaerobic glucose assimilation, was not observed either in Mutant 50 or Mutant 26 in contrast to the findings on PMS cyclic photophosphorylation. Thus it is possible that while PMS photophosphorylation may employ the same phosphorylation reactions as occur *in vivo*, the process does not involve similar electron transfer pathways. For comparison PMS photophosphorylation data obtained with a mutant deficient in P-700 (Mutant 8) and one deficient in Photosystem II activity (plastoquinone deficient) are included in Table II. The deletion of the reaction center of Photosystem I results in the complete loss of cyclic photophosphorylation activity but the loss of Photosystem II activity does not.

The lack of detectable bound cytochrome *b* in Mutant 26 also raises the question of the necessity for this cytochrome in cyclic photophosphorylation. However, the difficulties involved in the analysis for this type of cytochrome preclude any definite deductions at this time. Also, the total loss of both the lamellar-bound cytochrome types would probably invalidate any considerations on the function of cytochrome *b*.

Most of the data presented to this point have shown that the loss of cytochrome *f*, and perhaps of the *b*-type cytochromes, does not result in the loss of Photosystem I activity of isolated chloroplasts but does drastically inhibit Photosystem II activity (Table II). To determine if these mutants possess a functional Photosystem II analyses were made on their variable yield fluorescence. The variations in fluorescence yield of chlorophyll in intact photosynthetic systems have been related to the redox status of the hypothetical electron acceptor of Photosystem II, substance Q (ref. 24). Supposedly, the oxidized form quenches fluorescence while the reduced form (QH) does not; consequently, the steady-state level of fluorescence is a manifestation of the ratio of Q/QH. 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) is thought to prevent the

transfer of electrons from QH to the subsequent member of the electron transport system thus causing a decrease in this ratio and a higher fluorescence yield. Wavelengths of light which are preferentially absorbed by Photosystem II produce similar effects by causing excessive reduction of the fluorescence quencher. These interpretations have proved useful in a variety of recent studies on photosynthesis[24].

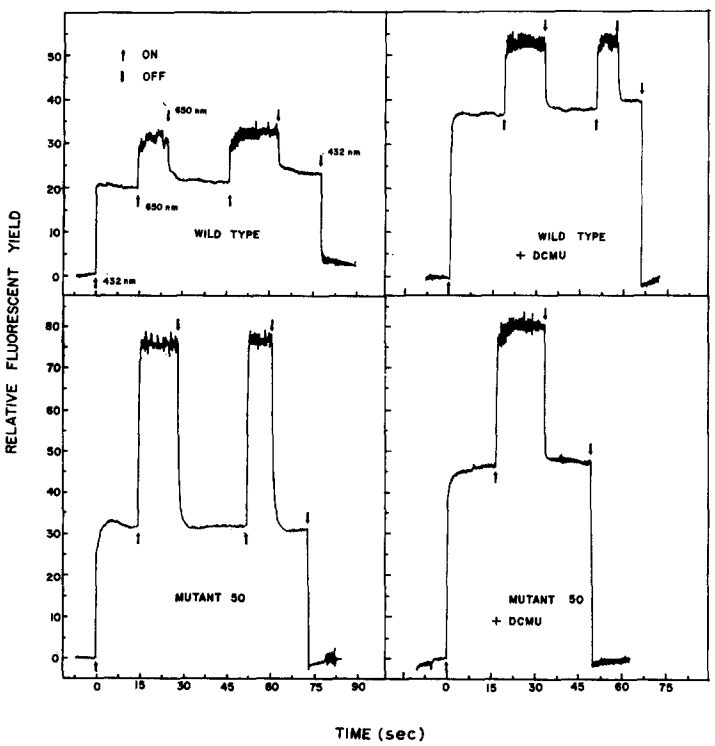

Fig. 5. Comparison of the influence of DCMU and Photosystem II light (650 nm) on the variable yield fluorescence of wild-type and Mutant 50 cells of *S. obliquus* D_3. 10 μl of cells were resuspended in 3 ml of fresh growing medium prior to fluorescence measurements. Gas phase, air. Temperature, 25°. Intensity of 436 nm light beam, 100 ergs·sec^{-1}·cm^{-2}. Intensity of 650 nm light beam, 200 ergs·sec^{-1}·cm^{-2}. Fluorescence monitored at 686 nm. DCMU concentration, 3 μM.

Data demonstrating the influence of DCMU and Photosystem II wavelength light on the fluorescence induced by a weak beam of 436 nm light are shown for Mutant 50 (Fig. 5). DCMU enhances the fluorescence yield by about 55 % and 650 nm light by 142 %. Similar data were obtained with cells of Mutants 8 and 26 but with Mutant 11, the Photosystem II mutant, an initial high yield of fluorescence was observed which was not influenced by the two treatments discussed above. We conclude that a functional Photosystem II exists but is not dedectable by conventional Hill reaction analysis because of interruption of the electron transport chain in Mutants 26 and 50. Our data support the view that cytochrome *f* is involved in a direct line of electron transport components coupling Photosystem I and Photosystem II. The progressively diminished Hill reaction rates of chloroplasts of Mutants 8, 50 and 11 indicate that, at least for algal systems, an intact electron transport

chain to the level of P-700 is required to sustain maximal rates of Hill oxidant reduction.

As suggested earlier, the biochemical block in Mutant 26 may be due to the lack of synthesis within the chloroplast of a protein component necessary for the binding of cytochrome *f* and *b*-type cytochromes. In Mutant 50 only the synthesis of cytochrome *f* is altered; all the other detectable soluble cytochromes are present in normal amounts. Since exogenously supplied cytochrome *c* 552 does not restore Photosystem II activity to chloroplast particles of this mutant, we must conclude, similarly to GORMAN AND LEVINE[25], that such a procedure does not allow specific access of the added soluble cytochrome to the functional site within the chloroplast or that an additional component required for reaction is absent either as a result of the mutation or as a consequence of preparatory procedures employed in isolating algal chloroplasts. However, it may be that cytochrome *c* 552 must be present in a form bound to the lamellar membranes for photosynthetic activity as suggested by BIGGINS[22].

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