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STUDIES ON THREE PHOTOSYNTHETIC MUTANTS OF SCENEDESMUS

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SUMMARY

Three mutants of Scenesdesmus D_3 designated 8, A', and 26 by Dr. Norman Bishop, were compared with the wild type strain with respect to their ability to fix $^{14}CO_2$ photosynthetically. Intact cells were also studied using the techniques of electron paramagnetic resonance (EPR) spectroscopy to identify the presence as well as the magnitude of the characteristic light-induced Signal I. Cell-free systems prepared from these same organisms were assayed for their competence to carry out photophosphorylation and photoreduction. The effect of several added co-factors in these systems was also studied including ferricyanide, phenazine methosulfate, ascorbic acid, and dichlorophenolindophenol (DCIP). Mutant 26 is able to carry out cyclic photophosphorylation, but is unable to photoreduce $NADP^+$. It has a normal EPR Signal I, and the lesion appears to be in the flow of electrons between Photosystem I and the pyridine nucleotide. Mutant A' is devoid of activity in Photosystem II. It is unable to carry out photoreduction unless electrons are provided from the DCIP-ascorbate couple. Mutant 8 is characterized by a unique ability to photoadapt its mutation. Mutant 8 cells grown in continuous light for approx. 30 days regain their photosynthetic ability and appear competent to carry out CO_2 fixation, photoreduction and photophosphorylation. Photosystem I activity and Signal I, absent in dark-grown cells is present in the light-grown organism. When such cells are returned to the dark they gradually deadapt and resume their mutant biochemical behavior. By treating dark-grown Mutant 8 cells with heat and a detergent, Triton X-100, Signal I appears. These data localize the lesion in Mutant 8 in the flow of the electrons in Photosystem I. It is postulated that alterations in the micro environment of Photocenter I induced by detergent or by light permit the flow of electrons through Photocenter I.

INTRODUCTION

The use of mutant species of various algae to elucidate the various biochemical and biophysical characteristics of the photosynthetic system has recently been reviewed in some detail[1,2]. A few mutants have been isolated and studied in some detail

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

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with respect to their ability to carry out CO_2 fixation[3], their complement of various electron carriers in the pathway[1], their specific concentrations of pigments operative in the photochemical processes[1], and the enzymes involved in the metabolic transformations[3].

The recent findings of LEVINE AND TOGASAKI[4] localized the sites of alteration in a series of Chlamydomonas mutants. The components lacking included the carboxylating enzyme as well as plastocyanine and cytochromes[5,6].

Scenedesmus mutants that we chose to study were induced by X-radiation and isolated by Dr. Norman Bishop, who generously sent them to us for this study. BISHOP[1,3] had already shown that these three mutants were incompetent to reduce CO_2 photosynthetically. They would grow only on a medium supplemented with glucose and yeast extract. They appeared normal in their chlorophyll content. Neither Mutant 26 nor Mutant A' could carry out O_2 evolution when provided with benzoquinone[1]. We have analyzed intact cells from normal and mutant species for their ability to fix $^{14}CO_2$ in the light as well as to exhibit the electron paramagnetic resonance (EPR) Signal I, as evidence of P700 function. Chloroplast particles were prepared and examined for their activity in photophosphorylation and photoreduction with the hope that specific lesions in these pathways could be elucidated.

METHODS

Mutants and wild type Scenedesmus D_3 were grown with gentle agitation in the dark using the glucose–yeast extract heterotrophic medium of BISHOP[1]. After a growth period of 10 days, the cells were harvested by centrifugation at 1000 $\times$ g for 10 min and washed twice by resuspending in 0.05 M Tris buffer (pH 7.5) and recentrifuged. Light-grown cells were prepared in the same medium; however, the flasks were exposed to continuous light at 1000 ft candles from a bank of fluorescent lamps. The cell cultures were transferred every 10 days to fresh medium and maintained in log-phase growth.

Tests for genetic reversion to wild type in the mutant cultures were made by plating an inoculum from the liquid dark-grown cultures on a series of solid agar slants containing either minimal or supplemented medium. These slants were placed in the light, grown for 7 days, and examined for presence of growth in the minimal medium. None was observed, while more than 1000 colonies grew on the enriched slants derived from more than 100 000 cells. It was concluded that there were no revertants. A control was carried out using wild type cells in which growth was normal on the minimal medium.

Techniques for $^{14}CO_2$ fixation

Cells were harvested by centrifugation at 1000 $\times$ g for 10 min. They were resuspended in 0.05 M Tris buffer (pH 7.5) and washed twice. The cells were then transferred to the steady-state medium of BASSHAM AND KIRK[7]. Radioactivity was provided as $KH^{14}CO_3$, 0.22 mM, containing 100 μC of ^{14}C prepared from high specific activity $Ba^{14}CO_3$ (ref. 8) obtained from Oak Ridge National Laboratories. The cell suspension was placed in a screw-cap glass vial in the presence of the radioactive bicarbonate and exposed to a light intensity of approx. 4000 ft candles in a Dubnoff metabolic shaking incubator maintained at 20°. Following 30 min of photosynthesis,

sufficient ethanol was added to make the medium 80% ethanol by volume. Ethanol extraction was facilitated by heating to 80° and maintaining this temperature for 5 min. This process stops the reaction and extracts chlorophyll as well as other low molecular weight products from the photosynthetic $^{14}CO_2$ fixation. Chlorophyll content was analyzed by the method of ARNON[9]. The total activity in the extract was measured by liquid scintillation techniques. The 80% ethanol extract was reduced in volume and the photosynthetic products separated by two dimensional paper-chromatographic procedures previously described[8]. Activity in each of the compounds was measured by cutting out the radioactive area in the two dimensional paper chromatogram and determining the activity by liquid scintillation techniques.

Photophosphorylation by chloroplast particles

Cell-free preparations were obtained using the Branson Heat Systems Sonifier (9 A for 2 min). Approx. 1 ml of packed cells were suspended in 5 ml of 0.1 M Tris buffer (pH 7.5), containing 5 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM GSH. The homogenate was cooled using a rosette cell placed in a salt–ice bath at —5°. Disruption of the cells was aided by the addition of 1.0 ml of glass beads, 0.5 mm in diameter. Following sonication, the extract was centrifuged at 1000 × *g* for 5 min to remove whole cells and debris. A chloroplast pellet was obtained by centrifuging the supernatant solution at 10 000 × *g* for 30 min. In experiments where sodium ascorbate was to be present, the suspending medium contained, in addition, 0.05 M sodium ascorbate during the course of the sonication procedure. The pellet was resuspended in 20 ml of 0.05 M Tris buffer (pH 7.5) containing 3 mM $MgCl_2$, 50 μM EDTA, and 50 μM GSH. In the ascorbate preparations, 0.02 M sodium ascorbate, was also present in the resuspending medium.

Photophosphorylation was carried out in an identical fashion to that described in our previous communications[10] except that the concentration of chlorophyll was considerably less that that present in the spinach preparations. Approx. 40–50 μg chlorophyll per 2.5 ml was present in the final reaction mixture. A final reaction volume of 2.5 ml contained 2.0 ml of chloroplast suspension, 1.0 μmole of ADP, 10 μmoles of $K_2H^{32}PO_4$ (containing approx. 30 μC of ^{32}P). In specific experiments one or more of the following compounds were added at the concentration indicated: 0.1 μmole PMS; 2.0 μmoles $NADP^+$; 2.0 μmoles $K_3Fe(CN)_6$; 60 μg ferredoxin; 0.1 μmole 2,6-dichlorophenolindophenol (DCIP); and 50 μmoles sodium ascorbate. Reactions were carried out for 5 min at 20° with illumination of 4000 ft candles. Photophosphorylation reactions were stopped by the addition of 0.5 ml of 50% trichloroacetic acid and the incorporation of ^{32}P into the organic fraction was measured by the techniques of AVRON[11].

Photoreduction of pyridine nucleotides

The procedure used the same chloroplast preparation as described above. Photoreduction was measured spectrophotometrically at 340 mμ using the techniques of SAN PIETRO AND LANG[12]. Experiments were carried out using 2 ml of the chloroplast suspension in a final volume containing 2.5 ml with 2.0 μmoles of $NADP^+$ and 60 μg of ferredoxin added. When present, sodium ascorbate was 50 μmoles and DCIP, 0.1 μmole. The duration of illumination was 5 min at 20° and 4000 ft candles. The reference cell in each case was the dark control. All data are expressed as light *minus* dark values of the $A_{340\ m\mu}$ of the NADPH formed.

Electron paramagnetic resonance spectroscopy

All measurements were made on a Varian EPR spectrometer at room temperature in a flat quartz cuvette, with techniques described elsewhere[13]. For the EPR measurements, harvested cells were washed twice in 0.1 M phosphate buffer (pH 7.2) containing 0.01 M KCl. Material was "heat treated" as follows: a 0.5-ml cell suspension was warmed in a water bath at 50° for 5 min. In order to observe maximum light-induced signal, bubbling with air for a few seconds after heating was necessary[14]. When Triton X-100 (Rohm and Haas) was used, it was added to a final concentration of 0.2%. EPR measurements were started within 5 min of heating and aerating, but could be repeated through several alternating traces made in the light and in darkness.

RESULTS

Photosynthetic fixation of $^{14}CO_2$

The rates and the patterns of distribution for $^{14}CO_2$ fixation in the three mutants are compared with the wild type in Table I. All values for the radioactivity present in a given compound or class of compounds is given as percent total radioactivity

TABLE I

RATES AND DISTRIBUTION OF $^{14}CO_2$ BY WHOLE CELLS GROWN IN LIGHT OR DARK

Experimental conditions: Photosynthetic $^{14}CO_2$ fixation for 30 min at 4000 ft candles. Light-grown cells maintained 30 days in light prior to harvest. Cells were suspended in the steady-state medium of BASSHAM AND KIRK[7]. $H^{14}CO_3$ was 0.22 mM containing 100 μC of ^{14}C. Distribution of ^{14}C activity in 80% ethanol extract is given in terms of percent of total counts.

Compounds	*Scenedesmus D_3 mutants*							
	Dark grown				*Light grown*			
	Wild type	*26*	*8*	*A'*	*Wild type*	*26*	*8*	*A'*
μmoles ^{14}C/mg chlorophyll per h	*85.0*	*0.51*	*3.0*	*0.6*	*65.0*	*0.71*	*72.0*	*0.290*
Diphosphates	5.3	4.3	4.7	11.8	9.7	5.6	10.9	3.2
Monophosphates	7.9	1.2	7.6	2.6	6.5	2.0	6.3	1.0
Total sugar phosphates	14.5	6.0	13.8	15.4	19.3	9.6	18.5	4.3
3-Phosphoglycerate	5.8	1.6	7.0	1.8	18.3	2.3	17.1	7.2
Total free sugars	5.7	8.2	4.7	12.0	5.5	2.3	6.4	1.7
Aspartate	32.8	41.0	22.3	21.5	18.4	31.4	19.5	36.0
Glutamate	6.3	19.8	7.3	27.3	3.5	20.0	3.1	26.0
Glycine–serine	2.6	1.0	3.8	1.8	4.3	0.2	6.3	0.4
Alanine	4.7	1.6	4.1	0.7	3.1	0.1	3.7	0.3
Total amino acids	50.0	67.1	40.0	54.2	30.7	42.5	34.0	70.2
Citrate	1.0	3.6	2.0	8.6	1.3	1.0	0.3	0.2
Malate	4.0	5.7	12.9	3.9	2.3	2.1	4.1	11.0
Glycerate	0.2	0.0	0.3	0.0	0.3	0.0	0.2	0.0
Glycolate	1.0	0.8	1.1	0.0	0.2	0.0	4.0	0.2
Total organic acids	7.3	10.2	17.8	13.0	4.8	6.1	8.7	15.2
Lipids	15.0	5.0	23.2	2.7	18.8	4.1	12.8	0.0

counted on the paper. Both light-grown and dark-grown cells are shown for comparison. The results indicate that there are considerable differences in the rate of $^{14}CO_2$ fixation by the wild type when compared with dark-grown mutants.

However, when grown continuously in the light for 30 days on heterotrophic medium, Mutant 8, but not Mutants A′ and 26, exhibited a marked increase in the rate of CO_2 fixation. In fact, the rate for light-adapted Mutant 8 was consistently slightly higher than wild type. In all cases, both in light and dark, there is no marked difference in the qualitative pattern of compounds observed.

In some mutants there may be rather large changes in lipids (*cf.* wild type with Mutant A′, or Mutant 8 with Mutant 26); nevertheless there is no striking absence of any important intermediate in the photosynthetic carbon cycle.

When light-adapted Mutant 8 cells are returned to the dark growth condition and subsequently tested for their ability to fix $^{14}CO_2$, it was found that they gradually lost this function (Table II). The results for wild type cells show an opposite effect.

Photophosphorylation

Results for photophosphorylation by chloroplast fragments from dark-grown cells are presented in Table III. All of the mutants show a decreased ability to photo-

TABLE II

DEADAPTATION OF PHOTOSYNTHETIC $^{14}CO_2$ FIXATION BY LIGHT-GROWN MUTANT 8 AND WILD TYPE CELLS

All values are in μmoles $^{14}CO_2$ fixed per mg chlorophyll per h. Experimental conditions were the same as Table I.

	Days in dark			
	0	*10*	*18*	*30*
Wild type	65.0	72.8	89.0	85.0
Mutant 8	72.0	17.6	4.4	3.1

TABLE III

PHOTOPHOSPHORYLATION BY SCENEDESMUS CHLOROPLAST PARTICLES

Experimental conditions: All cells were grown in heterotrophic media for 10 days in the dark. The sonicated chloroplast particles were suspended in a total of 2.5 ml containing: 100 μmoles Tris buffer (pH 7.5); 3 μmoles $MgCl_2$; 0.5 μmole EDTA; 0.05 μmole GSH; 1.0 μmole ADP; 10 μmoles P_i. Chlorophyll concn. was 40–50 μg. Additions were made at the following concns.: 0.1 μmole phenazine methosulfate; 2.0 μmoles $NADP^+$; 50 μmoles $K_3Fe(CN)_6$ and ascorbate. The reaction was carried out for 5.0 min at 20° with 4000 ft candles of illumination. Values are expressed as μmoles of P_i esterified per mg chlorophyll per h.

Additions	*Wild type*	*Mutant 26*	*Mutant 8*	*Mutant A′*
None	7.3	4.8	1.6	0.4
$NADP^+$	12.4	3.9	2.6	0.3
$NADP^+$ + ferredoxin	23.3	5.2	3.6	4.5
$NADP^+$ + ferredoxin + DCIP + ascorbate	18.0	3.3	1.3	18.5
Phenazine methosulfate	82.0	69.0	10.6	0.1
$[Fe(CN)_6]^{3-}$	29.0	—	11.0	1.8

phosphorylate compared with the wild type. The addition of $NADP^+$ and ferredoxin does not appreciably alter the rates of photophosphorylation in the mutants although a large and characteristic increase in the wild type rate was seen. The rates of cyclic photophosphorylation in Mutants 8 and A' are also quite low, although the rate obtained in the presence of phenazine methosulfate for Mutant 26 approaches the wild type rate. Mutant A' shows photophosphorylation only when Photosystem II is bypassed by the addition of an ascorbate–DCIP couple. These results confirm the apparent inability of Mutant A' to utilize water as an electron source as previously demonstrated by BISHOP[1].

The addition of ferricyanide shows a marked enhancement of photophosphorylation in Mutant 8. Even under these circumstances it is considerably below the comparable values obtained with wild type. The assay of photophosphorylation by chloroplast particles from light-grown Mutant 8 and wild type was complicated by difficulties in rupturing the cells. Preliminary experiments, however, indicate that significant photophosphorylation is carried out by the light-adapted mutant.

Photoreduction

The photoreduction of $NADP^+$ by chloroplast particles is shown in Table IV. In all of these experiments $NADP^+$ and ferredoxin were added at the concentration indicated. The three mutants exhibited marked inhibitions in the rate of pyridine nucleotide reduction. Photoreduction could be restored in Mutant 8 by the addition

TABLE IV

PHOTOREDUCTION OF $NADP^+$ BY SCENEDESMUS CHLOROPLAST PARTICLES

Experimental conditions were the same as Table III. Values are expressed as μmoles NADPH per mg chlorophyll per h.

Addition	*Wild type*	*Mutant 26*	*Mutant 8*	*Mutant A'*
$NADP^+$	30.0	0	1.6	0
$NADP^+$ + ferredoxin	114.8	5.1	35.6	6.1
$NADP^+$ + ferredoxin + DCIP + ascorbate	147.6	18.9	135.3	61.0

of an ascorbate–DCIP electron source. This should be contrasted with the photophosphorylation data where a very low level of phosphorylation was seen despite the very high rates of photoreduction observed in Table IV. Mutant 26 was relatively ineffective both in the presence and absence of the DCIP–ascorbate couple, while Mutant A' can be stimulated approx. 10-fold by the presence of the electron source. The ability of Mutant 8 to photoreduce $NADP^+$ in the presence of the DCIP–ascorbate couple without simultaneous photophosphorylation, suggested a direct photochemical interaction of Photosystem II with $NADP^+$.

The effect of temperature on photoreduction using chloroplast particles from Mutant 8 and wild type was studied. It is of particular interest that the apparent energy of activation of the electron flow seen in wild type is relatively high, when compared with Mutant 8, where a considerable amount of pyridine nucleotide is reduced even at 0° both in the presence and in the absence of the ascorbate–DCIP couple.

TABLE V

TEMPERATURE DEPENDENCE OF PHOTOREDUCTION

Experimental conditions were the same as Table III.

	Additions	*μmoles of NADPH per mg chlorophyll per h*	
	Temp.	*0°*	*20°*
Wild type	$NADP^+$	0	30.0
	$NADP^+$ + ferredoxin	0	114.0
	$NADP^+$ + ferredoxin + DCIP + ascorbate	9.0	147.0
Mutant 8	$NADP^+$	0	1.6
	$NADP^+$ + ferredoxin	13.8	35.6
	$NADP^+$ + ferredoxin + DCIP + ascorbate	24.8	135.0

EPR spectroscopy

The characteristic spectrum for the rapid light-induced EPR Signal I in dark-grown wild type cells is shown in Fig. 1. This signal is absent in dark-grown Mutant 8 cells (Fig. 1). After Mutant 8 cells have been grown heterotrophically for 30 days in continuous light, the characteristic Signal I appears (Fig. 1). There is considerable

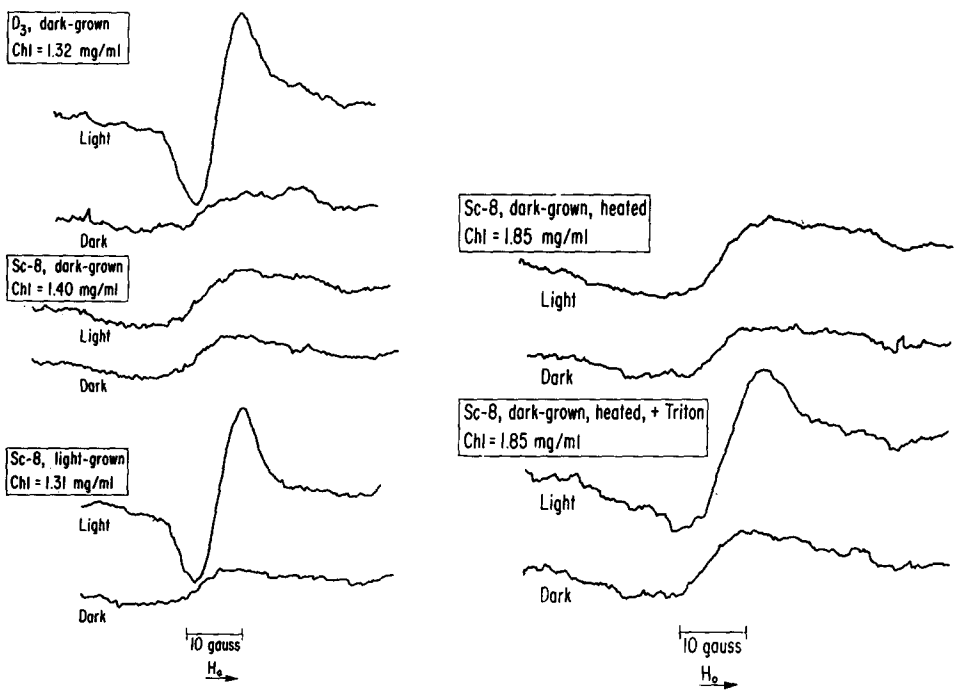

Fig. 1. EPR spectra of light- and dark-grown wild type (D_3) and Mutant 8 (Sc-8).

Fig. 2. Effect of heating and Triton X-100 on EPR signal from Mutant 8 (Sc-8). The cells were heated to 60° for 4.0 min and treated with 0.2 % Triton X-100.

evidence to indicate that the magnitude of the signal corresponds to the turn-over of electrons through the Photocenter I, P700 (ref. 14). Mutants A′ and 26 have Signal I equivalent in all respects to that of the dark-grown wild type. However, Signal II which is correlated with O_2-evolving ability[15] was absent in Mutants A′ and 26.

When dark-grown Mutant 8 cells were warmed to 60° for 4 min and made 0.2% by wt. in Triton X-100, within the time required to return the cuvette to the spectrophotometer and reilluminate the cells, Signal I appears and is qualitatively identical to that observed in normal cells (Fig. 2). When the light is turned off Signal I disappears in the Triton-treated cells. Signal I in wild type cells is also increased by the heat-*plus*-Triton treatment. Heating alone would not restore Signal I in Scenedesmus Mutant 8.

DISCUSSION

On the basis of the results presented above, it is possible to speculate on the nature and localization of the lesions in the three mutants studied. Mutant 26 appears to have an impaired flow of electrons into Photosystem I from Photosystem II. Thus Mutant 26 will carry out an effective cyclic photophosphorylation in the presence of phenazine methosulfate, but under none of the conditions tested is it able to effectively reduce $NADP^+$. Only in the presence of the DCIP–ascorbate couple is there appreciable $NADP^+$ reduction observed. There may also be a lesion in one of the electron carriers near Photosystem II.

Mutant A′ has all of the characteristics of an organism devoid of Photosystem II (ref. 1). Only by introducing electrons into the chain of carriers using the DCIP–ascorbate couple can a reasonable rate of photoreduction and photophosphorylation be achieved. These data agree very closely with the results using benzoquinone with intact cells[3].

Of the three mutants reported here, Mutant 8 is the only one able to photoadapt over a long period of exposure to illumination. That this is not caused by genetic reversion to a wild type cell or by a contaminant whose growth is at such a rate as to completely overgrow the mutant, was demonstrated by the testing of the light- and dark-grown cells for their ability to grow on minimal medium. The evidence for this photoadaptation is provided by all parameters tested including photosynthetic CO_2 fixation, photophosphorylation and photoreduction.

It has been proposed that the lesion in Mutant 8 is the absence of the reaction center, P700, which is not optically detectable. However, both EPR and optical methods of detecting P700 are based on the transfer of an electron from the reaction center to an acceptor, resulting in an absorbance change due to bleaching at 700 mμ, or the EPR signal due to P700. Mutant 8 has a strong fluorescence peak at 730 mμ (ref. 16). One must conclude that either P700 is not the source of this band, or that P700 is the source of it and just not functioning photosynthetically, making it invisible optically or by microwave spectroscopy. Similar reasoning can be applied to the fluorescence emission spectrum in Mutant 8 which appears to be as polarized as the one from wild type[17]. A blockage of electron flow to the reaction center would account for all these observations, but so would a block preventing the transfer of an electron from P700 to the acceptor. The fact that excess ferricyanide does not elicit an EPR signal, nor bleach the 700-mμ absorption band would argue for a model in

which the reaction center was for some reason inaccessible even to exogenous oxidizing agents. CLAYTON[18] has suggested that the photoreactive center is chlorophyll in a special environment. Our results suggest that heating *plus* detergent treatment alter the environment in a way which facilitates electron flow. Heat alone, which slows down signal decay rate in wild type, and results in a larger signal, does not greatly alter the signal size in Mutant 8.

The inability of ferricyanide to induce an EPR signal in Mutant 8 while inducing appreciably phosphorylation is probably due to its ability to remove electrons as they flow from Photosystem II through the non-cyclic chain at a point prior to cytochrome (KOK AND DATKO[19]). In the ferricyanide experiments we are seeing only the operation of the non-cyclic system, although some enhancement is observed upon the addition of phenazine methosulfate. It thus appears that the block is not complete. That Mutant 8 is otherwise competent to carry out photoreduction is shown upon the addition of the DCIP–ascorbate couple where rates equivalent to wild type are obtained. In this instance the energy of activation of photoreduction is considerably altered. It is tempting to explain these results on the basis of the interesting experments of KOK AND DATKO[19] who demonstrated that Mutant 8 could reduce methyl viologen in high light intensity at appreciable rates. In the presence of electrons provided by the ascorbate–DCIP couple and the photoreducing power provided by Photosystem II, there could be a reduction of either ferredoxin or $NADP^+$. This hypothesis is in keeping with the interesting observations of VERNON *et al.*[20] who have shown a direct reduction of $NADP^+$ with electrons from ascorbate and DCIP when chlorophyllin *a* is present as a photocatalylist. The relatively low energies of activation observed here are in keeping with the photochemical rather than enzymatic nature of this process. Recently BERTSCH *et al.*[21] looking at the delayed emission of Mutant 8 showed a very slow decay of this emission indicating little electron flow from Photosystem II to Photosystem I.

The results presented in this paper more closely define the sites of the biochemical lesions in three photosynthetic mutants of Scenedesmus. Unfortunately, we are unable to demonstrate the precise single enzyme or electron carrier which is absent in any of the mutant strains. Of particular interest is the important interrelationship that structure and function may play in Mutant 8. Alteration in the lipid environment by detergent can lead to appearance of Signal I and would suggest that structural integrity of the chloroplast, particularly the lipid environment in which the carriers exist, plays a distinct role in the solid state photochemistry and electron transport. ROSENBERG[22] reached similar conclusions regarding the importance of the lipid phase of the membrane systems of the chloroplasts. In many respects these mutants are analogous to the pleiotropic mutants observed in many microorganisms. Experiments are underway to elucidate the nature of the lipid changes involved to correlate them with the biochemical phenomena observed.

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