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# Isolation of Mutants of Euglena gracilis With Impaired Photosynthesis[1]

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*Abstract.* **Four mutant strains of *Euglena gracilis* have been isolated after treatment of wild type cells with ultraviolet light or the chemical mutagen nitrosoguanidine. None of the mutants is capable of autotrophic growth or photosynthetic carbon dioxide fixation.**

**The mutant strains contain normal amounts of the enzymes of the reductive pentose phosphate cycle and are qualitatively similar to the wild type in pigment composition, but are unable to carry out the Hill reaction (light induced reduction of 2,6-dichlorophenol indophenol). Isolated mutant plastids cannot photoreduce NADP with water as the electron donor but can carry out this reaction when the electron donating system is ascorbate and 2,6-dichlorophenol indophenol. Whole cells of the mutants show the light induced oxidation of cytochrome *f* by light reaction I but are unable to bring about cytochrome *f* reduction by light reaction II. The mutants appear to be blocked at or near light reaction II in the photosynthetic electron transport chain.**

**The mutants may represent alterations of the chloroplast genome since the mutation isolation was carried out under conditions where chloroplast viability was severely impaired, but cell viability was unaffected.**

Treatment of whole cells of *Euglena gracilis* with a variety of mutagenic agents including ultraviolet light (9), nitrofurans (12), nitrosoguanidine (13), nalidixic acid (11), streptomycin (17) and many others (*cf.* 21) gives rise to white, permanently bleached strains. Many of these agents seem to prevent the replication of the chloroplast (or proplastid in dark grown cells) without affecting cell division, so that the plastid genome is irreversibly deleted from the cell during division. As part of an investigation of the effects of ultraviolet light and nitrosoguanidine on *Euglena* cells, we noticed that many of the cells which were not permanently bleached by intermediate doses of the mutagenic agents gave rise to pale green colonies containing much less chlorophyll than normal green wild type colonies. A detailed biochemical investigation of several of these pale green mutants indicates that these strains are incapable of autotrophic growth because of impairment of the photosynthetic apparatus. All of the mutants we have investigated seem to be blocked at or near system II of the photosynthetic electron transport chain.

## Materials and Methods

*Organism and Culture Conditions.* *Euglena gracilis* var. *bacillaris* strain Z (Pringsheim) was grown on a rotary shaker in defined heterotrophic medium (pH 3.5) (5) at 26°. Wild type cultures were illuminated with 30 watt Sylvania warm white fluorescent bulbs; the light intensity at the surface of the growth flask was 500 foot-candles. The photosynthetic mutants were cultured in the same medium but the light intensity was reduced to 200 foot-candles by covering the flasks with 2 layers of cheesecloth. For photosynthetic growth of wild type cells the medium of Lyman and Siegelman (10) was used.

*Mutation Induction.* Ultraviolet irradiation and plating were carried out as described previously (9).

*N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin. Washed *Euglena* cells ($10^6$/ml) were suspended in 50 mM citrate buffer (pH 5.0) containing nitrosoguanidine (50 μg/ml) for 20 minutes. At the end of the incubation period, the cells were diluted with phosphate buffer and plated onto *Euglena* agar (Difco) with a 3 ml top layer of the same medium. The plates were incubated in the light for 1 week at 25°. The treatment killed less than 5 % of the cells as determined by plate count. Approximately 60 % of the surviving cells gave rise to white colonies; the remaining 40 % consisted of a mixture of normal green colonies and pale green colonies.

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Revertants were isolated by plating cells of the pale green mutants onto inorganic plates (approximately $10^6$ cells/plate) on which the mutant cells could not grow. Revertants appeared as green colonies after incubation in the light for a week.

*Assay of Photosynthetic Reactions.* $CO_2$ fixation by whole cells was measured in 20 ml test tubes. Each tube contained whole cells equivalent to 5 to 10 $\mu$g chlorophyll in 50 mM phosphate buffer (pH 7.5) in a total volume of 1.0 ml. To start the reaction $NaH^{14}CO_3$ (5 $\mu$curies, 5 $\mu$moles) was added and the tube was incubated at 25° at a light intensity of 2000 foot-candles (saturating). An unilluminated control was included to correct for dark fixation. The reactions were terminated by adding 0.3 ml 0.5 N HCl and aliquots were counted. Photosynthesis was linear with respect to time and was proportional to the quantity of cells used in the assay.

Soluble extracts were prepared by sonication with a Branson ultrasonifier (10 kc) for 60 seconds. The extracts were clarified by centrifugation for 30 minutes in a refrigerated Servall centrifuge at 25,000 × *g*. Soluble protein was determined by the biuret method (4).

Ribulose-1,5-diP carboxylase, glyceraldehyde-3-P dehydrogenase, fructose-1,6-diP aldolase, fructose-1,6-diphosphatase (alkaline), ribose-5-P isomerase and ribulose-5-P kinase were assayed as described by Russell and Gibbs (18, 19). All reactions were linear with time and were proportional to the amount of extract.

Chloroplast fragments were prepared by sonicating whole cells for 30 seconds. Whole cells were removed by centrifugation at 1000 × *g* for 5 minutes. The supernatant of the low speed spin was centrifuged for 10 minutes at 5000 × *g* to sediment the chloroplast fragments. Chlorophyll concentrations were determined by Arnon's method (1). Absorption spectra of pigment solutions were taken with a model 14 Cary recording spectrophotometer.

The Hill reaction with 2,6-dichlorophenol indophenol (DCPIP) as the electron acceptor was measured as described by Levine and Smillie (7). The light induced reduction of NADP was determined according to Katoh and San Pietro (6). Each reaction mixture contained in a final volume of 1.0 ml: 50 $\mu$moles phosphate buffer (pH 6.0), 0.2 $\mu$mole NADP, 0.05 $\mu$mole reduced *Euglena* cytochrome $f_{552}$, saturating amounts of spinach ferredoxin and *Euglena* chloroplast fragments equivalent to 10 to 15 $\mu$g of chlorophyll. *Euglena* cytochrome $f_{552}$ was prepared according to the method of Perini *et al.* (16). Ferredoxin was purified from spinach leaves (16). NADP photoreduction by DCMU inhibited chloroplast fragments with ascorbate-DCPIP as the electron donating system was determined in reaction mixtures similar to the above, supplemented with 0.01 $\mu$mole DCMU, 7.5 $\mu$moles ascorbate and 0.02 $\mu$mole DCPIP.

Cytochrome *f* concentrations in the wild type and mutant were measured according to Perini *et al.* (16). The insoluble electron transport component, cytochrome $b_{561}$, was assayed in acetone powders of *Euglena* cells (16).

Double-beam spectrophotometry was carried out on whole cells using an instrument constructed by Dr. John Olson (14). The instrument has 2 actinic beams and can be used to study the interaction of the 2 light reactions of the photosynthetic electron transport chain.

## Results

Twenty-five pale green mutants were isolated from experiments in which wild type *Euglena* was treated with ultraviolet light or the chemical mutagen nitrosoguanidine. Four of these were selected for further study. Table I summarizes information about the mutant strains. Since the results were identical for each of these strains, the data presented in this paper are taken from experiments with $P_4$, pale green mutant 4.

Table I. *Some Characteristics of Photosynthetic Mutants of* Euglena gracilis

Chlorophyll concentrations are given as mg chlorophyll/cell. Under the same conditions of growth wild type cells produce 5 to 10 × $10^{-9}$ mg chlorophyll/cell.

| Mutant no. | Mutagen | Reversions | Chlorophyll/cell |
|---|---|---|---|
| $P_4$ | Ultraviolet | — | 2.9 × $10^{-9}$ |
| $P_7$ | Nitrosoguanidine | + | 2.0 × $10^{-9}$ |
| $P_8$ | Nitrosoguanidine | — | 1.9 × $10^{-9}$ |
| $P_9$ | Nitrosoguanidine | + | 3.6 × $10^{-9}$ |

Cells of $P_4$ are unable to grow autotrophically in vitamin supplemented salts media under which condition the wild type organism divides with a doubling time of 10 to 12 hours (fig 1). The mutant cells grow rapidly in both the light and dark in a medium containing glutamate and malate; in this medium the doubling time is similar to that of the wild type.

In figure 2 it can be seen that cells of $P_4$ are unable to carry out photosynthesis, as measured by the rate of incorporation of $NaH^{14}CO_3$. Wild type *Euglena* fixes $CO_2$ at a high rate in the light but the mutant shows little if any light stimulated $CO_2$ uptake under the same conditions. Similar results were obtained in experiments in which photosynthesis was measured as light dependent $O_2$ evolution in the Warburg apparatus. The rates of dark $CO_2$ fixation are similar in both the mutant and wild type (fig 2).

The inability of $P_4$ cells to carry out normal photosynthesis is not due to the lower pigment content *per se*. The mutant does not fix $CO_2$ at light intensities which are saturating for wild type *Euglena* (fig 3). In addition, we have isolated other pale green mutants with similarly low amounts of chlorophyll which are capable of normal photosynthesis.

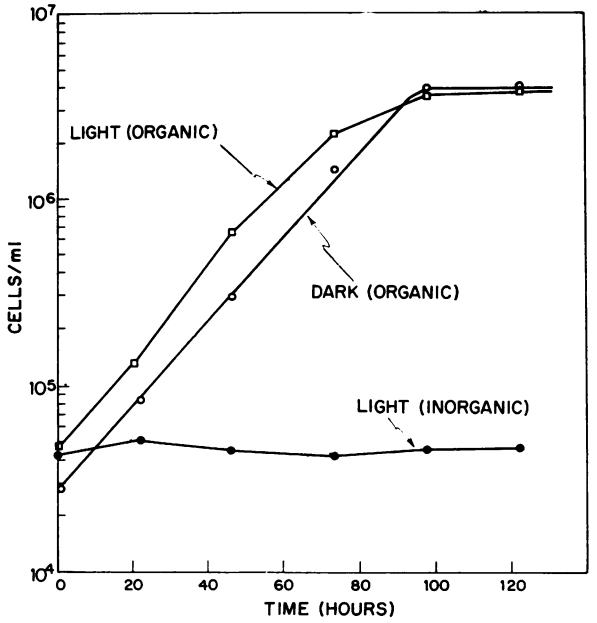


FIG. 1. Growth of mutant $P_4$ in organic and inorganic media. The number/ml of culture is plotted *versus* time in hours. -●-●-●- represents growth in organic medium in the light; -○-○-○- represents growth in organic medium in the dark; -□-□-□- represents growth in organic medium in the light.

The inability of $P_4$ to incorporate $^{14}CO_2$ at low light intensities (fig 3) eliminates the possibility that the mutant might contain light sensitive plastids which are photoinactivated at higher light intensities. Examination of $P_4$ cells by fluorescence microscopy indicated the presence of 8 to 12 plastids.

The photosynthetic apparatus of green plants consists of several component parts, the impairment of any one of which would produce a cell unable to carry out photosynthesis. A systematic analysis was carried out on wild type and mutant *Euglena* with respect to 1) pigment content, 2) enzymes of the reductive pentose phosphate cycle, and 3) the photosynthetic electron transport system.

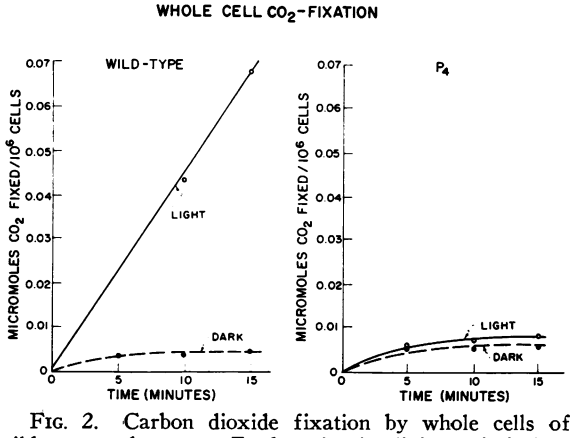


FIG. 2. Carbon dioxide fixation by whole cells of wild type and mutant *Euglena* in the light and dark.

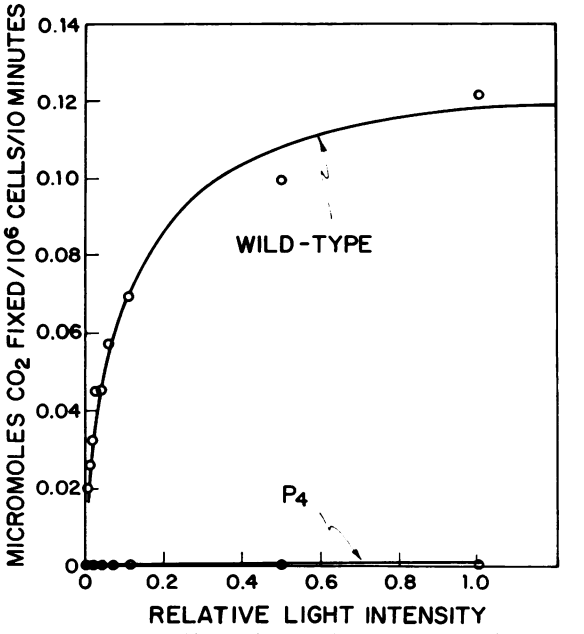


FIG. 3. The effect of light intensity on carbon dioxide fixation in wild type and mutant *Euglena*. The light intensity was varied by placing tubes with the reaction mixture at various measured distances from a white light source such that the relative light intensity "1.0" was equal to 2000 foot-candles. The points are corrected for dark fixation.

*Pigmentation.* Absorption spectra of acetone extracts of equal numbers of wild type and mutant cells are presented in figure 4. It can be seen that the chlorophyll content of $P_4$ is approximately 40 % that of the wild type. The carotenoid content of $P_4$ cells is also reduced as indicated by light absorption in the region 470 to 480 nm. Thin layer chromatography of acetone extracts indicated that $P_4$

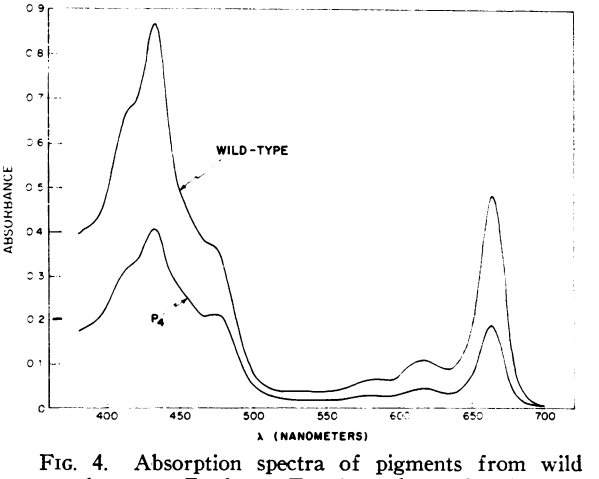


FIG. 4. Absorption spectra of pigments from wild type and mutant *Euglena*. Equal numbers of cells were extracted in 80 % acetone and the spectra recorded on a Cary spectrophotometer.

Table II. *Comparison of Enzyme Activities in Extracts of Wild Type and Mutant* Euglena gracilis

Ribulose-1,5-diP carboxylase was measured in the following reaction mixture: *Euglena* extract, $NaH^{14}CO_3$ (10 μmoles, 5–10 μcuries), $MgCl_2$ (20 μmoles), EDTA (0.1 μmole), GSH (12.5 μmoles) and 0.25 μmole ribulose-1,5-diP in a total volume of 2.0 ml. The reactions were run for 10 minutes, terminated with 0.5 N HCl and assayed for counts fixed. Ribose-5-P isomerase, ribulose-5-P kinase and ribulose-1,5-diP carboxylase were measured simultaneously by incubating the above reaction mixture with ATP (1 μmole) and ribose-5-P (2 μmoles) instead of ribulose-1,5-diP. The rates of $CO_2$ incorporation in these 2 assays were identical.

NADP dependent glyceraldehyde-3-P dehydrogenase activity was measured in the reverse direction according to the method of Wu and Racker (*23*).

Fructose-1,6-diP aldolase was measured by coupling with triose-P isomerase and α-glycerol-P dehydrogenase (19).

Ribose-5-P isomerase, epimerase and transketolase were measured simultaneously in the following reaction mixture: *Euglena* extract; tris buffer (pH 8.0), 20 μmoles; excess commercial glyceraldehye-3-P isomerase and α-glycerophosphate dehydrogenase (Sigma); NADH, 0.15 μmole and ribose-5-P, 5 μmoles in a total volume of 1.0 ml. The reaction was followed in the Cary by measuring the decrease of absorption at 340 nm.

| Enzymes of reductive pentose phosphate cycle | Wild type | $P_4$ |
|---|---|---|
| | *μmoles substrate reacting per hr per mg soluble protein* | |
| Ribose-5-P isomerase, Ribulose-5-P kinase, and Ribulose-1,5-diP carboxylase | 0.89 | 0.88 |
| Glyceraldehyde-3-P dehydrogenase (NADP dependent) | 32 | 23 |
| Fructose-1,6-diP aldolase (Class I) | 8.9 | 6.8 |
| Fructose-1,6-diphosphate (alkaline) | 8 6 | 8.1 |
| Ribose-5-P isomerase, epimerase, and transketolase | 2.1 | 1.9 |

cells contain both chlorophylls *a* and *b* and all of the carotenoids which are present in the wild type; the total amounts of all of the pigments are reduced.

*Enzymes of the Reductive Pentose Phosphate Cycle.* A comparison of the activities of enzymes of the reductive pentose phosphate cycle is given in table II. Soluble extracts of $P_4$ were found to contain substantial amounts of the photosynthetic carboxylating enzyme, ribulose-1,5-diP carboxylase. When supplied with ribulose-1,5-diP and $NaH^{14}CO_3$, the mutant extracts incorporated radioactivity into acid soluble compounds at a high rate.

Other enzymes of the cycle including glyceraldehyde-3-P dehydrogenase (NADP dependent), fructose-1,6-diP aldolase and fructose-1,6-diphosphatase were measured with mutant extracts (table II). The qualitative presence of ribose-5-P isomerase, ribulose-5-P kinase, transketolase, epimerase, and 3-P-glycerate kinase was detected in extracts of $P_4$. It does not seem likely that a deficiency in the reductive pentose phosphate cycle is responsible for the inability of $P_4$ to carry out normal photosynthesis.

*Electron Transport Reactions.* Chloroplast fragments isolated from wild type *Euglena* were found to carry out the light dependent reduction of DCPIP (Hill reaction) at a high rate (fig 5). Aliquots of fragments containing equivalent amounts of chlorophyll isolated from $P_4$ plastids were unable to perform this reaction (fig 5). Similar results were obtained when the reaction was measured as light dependent $O_2$ evolution by chloroplast fragments with the Warburg apparatus. Attempts to measure the Hill reaction manometrically with whole cells using a variety of oxidants (potassium ferricyanide, DCPIP and *p*-benzoquinone) were unsuccessful.

Measurements of the photoreduction of NADP by isolated chloroplast fragments of *Euglena* are complicated by the loss of cytochrome *f* during the plastid isolation procedure (6, 15). Katoh and San Pietro (6) have recently shown that the addition of cytochrome *f* to the reaction mixture restores

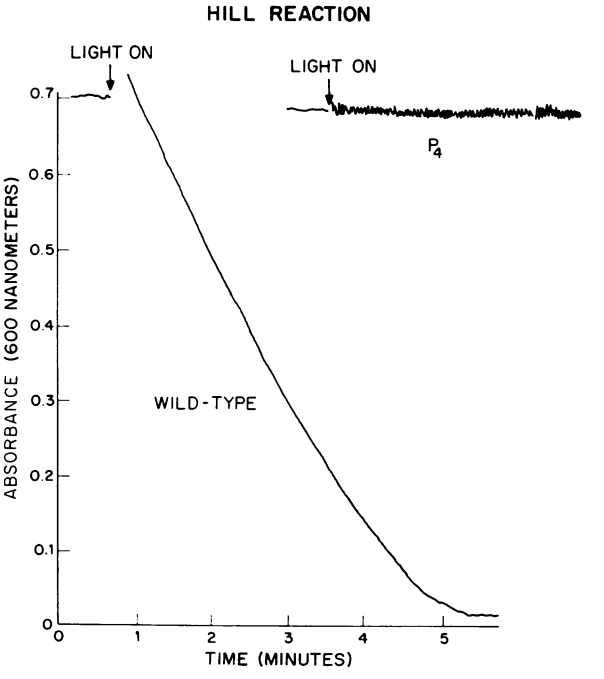


FIG. 5. Hill reaction activity of chloroplast fragments of wild type and mutant *Euglena*. The Hill reaction was measured in the Cary spectrophotometer as the disappearance of absorption at 600 nm due to the reduction of 2,6-dichlorophenol indophenol. The reaction mixture contained chloroplast fragments (3–4 μg chlorophyll) and the following in μmoles: phosphate buffer (pH 6.8), 10; KCl, 5; and 2,6-dichlorophenol indophenol, 0.05 in a total volume of 0.8 ml. The infrared lamp was used as the source of actinic and measuring light.

NADP photoreducing capacity. Our attempts to measure the overall photosynthetic electron transport chain in the presence of *Euglena* chloroplast fragments, spinach ferredoxin and *Euglena* cytochrome *f* gave rates of 20 to 30 μmoles NADP reduced per hour per mg chlorophyll for the wild type and no detectable activity for chloroplasts from $P_4$. This confirms the presence of a block in the electron transport chain in $P_4$. Very low rates of NADP photoreduction were measured with both wild type and $P_4$ plastids in the presence of 10 μM DCMU with ascorbate and DCPIP as the electron donor. The highest rates we measured were 10 to 15 μmoles NADP reduced per hour per mg chlorophyll for both the wild type and $P_4$. This finding demonstates that light reaction I functions in the mutant strain.

Analysis of electron transport components indicated the presence of at least 2 electron carriers in both mutant and wild type *Euglena*. Cytochrome $f_{552}$ and cytochrome $b_{561}$ were detected in $P_4$ as well as in the wild type.

Since fairly substantial amounts of cytochrome *f* were detected in $P_4$ cells, it was possible to investigate the interaction of the 2 light reactions of photosynthesis using double-beam spectrophotometry. Figure 6 shows the state of oxidation of cytochrome *f* in whole cells of the wild type under a variety of conditions. Illumination of wild type cells with light of wavelength 700 nm (absorbed almost exclusively by light reaction I) brought about a decrease of absorption at 552 nm. A light minus dark difference spectrum of this change demonstrated clearly that this represented the light induced oxidation of cytochrome *f* (fig 7A). Light of wavelength 660 nm (absorbed by both light reactions I and II) brought about a small oxidation of the cytochrome but the final steady state was largely reduced (fig 6A). An intermediate state of cytochrome *f* oxidation could be produced by illuminating the cells with both 660 nm and 700 nm light; any steady state level of oxidation could be obtained by adjusting the light intensities of the 2 actinic beams. In all experiments system I oxidized cytochrome *f* and system II reduced the cytochrome. These results are similar to those described for *Euglena* by Olson and Smillie (15).

Figure 6B shows the effect of 19 μM DCMU on the light induced changes in wild type *Euglena*. In the presence of a very weak system I light, 660 nm light was unable to reduce *f* to any appreciable extent. This confirms the well-known effects of the herbicide on system II of the photosynthetic electron transport chain.

The interaction of the 2 light reactions on the state of cytochrome *f* oxidation in $P_4$ is given in figure 6C. System I light brings about a rapid oxidation of *f* but system II is unable to reduce *f*, even when 700 nm light is turned off, and cytochrome *f* is kept oxidized by the 660 nm light absorbed by system I. The results with $P_4$ are entirely com-

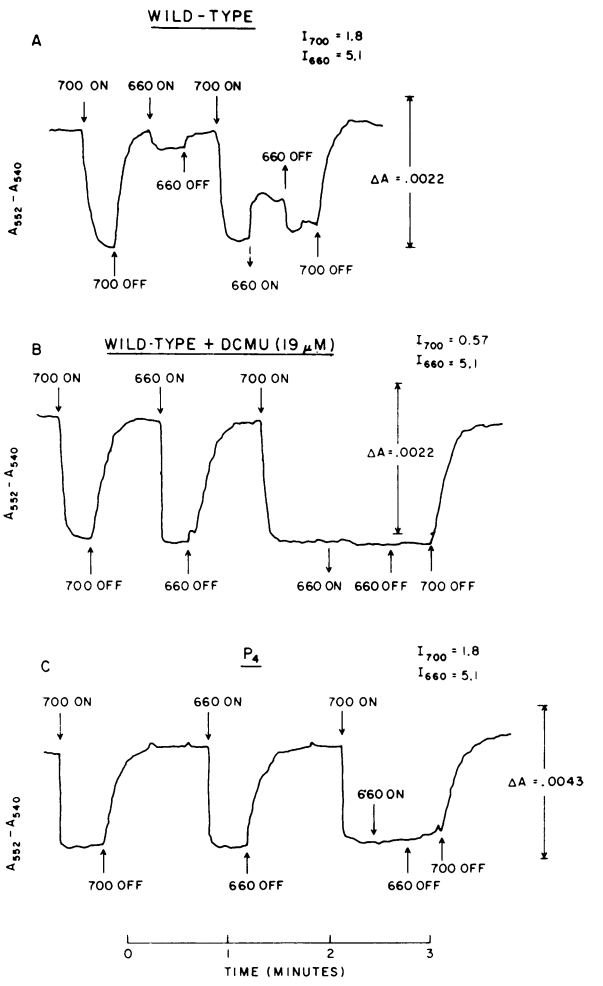


FIG. 6. Oxidation and reduction kinetics of cytochrome *f* in whole cells of wild type and mutant *Euglena*. Cells were illuminated in a Chance-type double-beam spectrophotometer in which the measuring beams were deflected vertically through the cuvette to minimize changes in transmission due to settling of cells (14). Monochromatid actinic light (either 660 nm or 700 nm) was projected vertically through a half-silvered mirror into the cuvette from a 50 × 50 mm slide projector and the following added: a 2 cm water layer, a Bausch and Lomb interference filter and a Corning red cut-off filter (for 700 nm, a #700 B & L filter and a Corning CS 2-64; for 660 nm a B & L #660 and a Corning CS 2-62.) The actinic light intensity was monitored with an RCA photodiode calibrated against a precalibrated Epply 12 junction thermopile in the sample position. The light intensity ("I") is expressed as nanoeinsteins per $cm^2$ per sec. Two slide projectors, modified as above, were set so that 2 actinic beams could illuminate the sample simultaneously. 3-(3,4-dichlorophenyl) 1,1-dimethyl urea (DCMU) in ethyl alcohol was added to give a final concentration of 19 μM. A) Light induced spectral changes ($A_{552}$–$A_{540}$) in wild type *Euglena*; B) Light induced spectral changes ($A_{552}$–$A_{540}$) in wild type *Euglena* poisoned with 19 μM DCMU. C) Light induced spectral changes ($A_{552}$–$A_{540}$) in $P_4$.

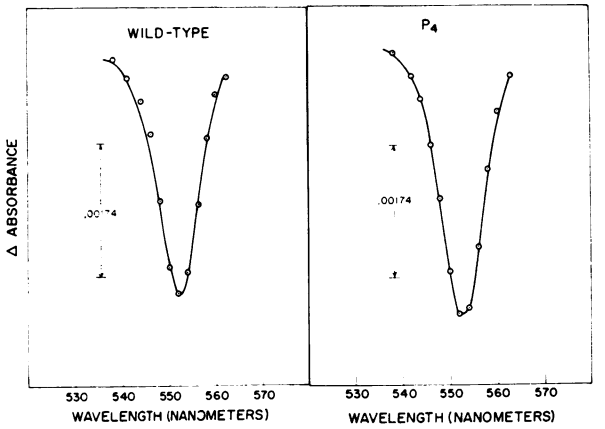


FIG. 7. Light minus dark difference spectra of wild type and mutant *Euglena*. A) Light induced spectral changes at various wavelengths *versus* 540 nm are plotted for wild type cells. B) The same measurements are plotted for $P_4$.

parable to the results obtained with DCMU-poisoned wild type. $P_4$ plastids contain an active photosystem I but are unable to bring about cytochrome *f* reduction by system II. The difference spectrum for the light induced change is given in figure 7B.

In both $P_4$ and DCMU-treated wild type, cytochrome *f* became reduced again when the actinic beam was turned off (figs 6B and 6C). This may indicate the presence of a cyclic electron flow mechanism which brings about the reduction of cytochrome *f* even when system II is not functioning, or may suggest the presence of a pool of internal reductant. Attempts to exhaust the possible internal pool by successive photooxidations were unsuccessful.

It appears that $P_4$ cells contain system II pigments, since these cells emit intense red fluorescence at 685 nm when irradiated with 437 nm light (Lyman and Russell, unpublished experiments). The fluorescence yield in $P_4$ is 5 to 6 times greater than in wild type *Euglena*. A substantial fraction of this may be due to the reemission of light absorbed by pigment system II.

## Discussion

Many of the mutagenic agents which have been tested on *Euglena gracilis* produce mutant strains in which the chloroplasts (and chloroplast DNA) have been irreversibly lost from the cell (3); these strains have never been observed to revert to wild type (21). Our results indicate that it is possible to obtain mutants of *Euglena* in which the genetic alteration is probably a single gene mutation rather than a large deletion of genetic material. The finding that several of the pale green mutants revert to wild type represents one of the first reports of genetic reversion in this organism.

The isolation of mutants of *Euglena* is rather unexpected since there appear to be 6 to 8 nuclear genomes (21) and 20 to 30 cytoplasmic (chloroplast) genomes (9) in the organism; the probability of inducing the same mutation simultaneously in all of the genomes is remotely small. The very high sensitivity of chloroplast replication to various physical and chemical agents suggests the following hypothesis to account for the production of the mutants. The pale green strains might represent clones derived from cells in which all but one of the chloroplast genomes were prevented from replicating by the mutagenic treatment and a mutation was induced in the 1 surviving genome. This mutated genome could then have multiplied several times to produce a cell in which all of the chloroplasts carried the mutation. Our results are compatible with this hypothesis. The possibility that a single chloroplast genome can reconstitute an entire set of genomes in *Euglena* is suggested but not proven by target analysis of dose-response curves for ultraviolet inactivation of chloroplast replication (9). It should be emphasized that the mutagenic treatments employed in our experiments had no effect on cell viability but had substantial effects on plastid replication. Definitive proof that the pale green strains are single gene mutants and identification of the site (genome) within the cell which is altered must await the discovery of a sexual system in *Euglena* (if such exists).

The evidence reported here suggests that $P_4$ (and the other mutants we have studied) are blocked at or near light reaction II of the photosynthetic electron transport chain. The inability of chloroplast fragments of $P_4$ to carry out the photoreduction of DCPIP and NADP from water and the evidence derived from double-beam spectrophotometry on whole cells of $P_4$ support this conclusion. The finding that $P_4$ plastids can photoreduce NADP from DCPIP and ascorbate and that whole cells of the pale green mutant can photooxidize cytochrome *f* support the notion that light reaction I functions normally in this strain. Further investigations are in progress to delineate the exact nature of the lesion in the mutant. Since there is very little known about the interaction of pigment system II with the photosynthetic electron transport chain, the *Euglena* mutants should be valuable experimental tools in elucidating the details of photosynthetic electron transport [*cf.* Levine (8) and Bishop (2)].

Our results suggest that chloroplast development is gratuitously induced in *Euglena;* proplastids of dark grown cells of $P_4$ develop into chloroplasts upon illumination even though the plastids are photosynthetically incompetent. This result confirms the recent findings of Schiff, Zeldin, and Rubman (22).

Although it is not known why a lesion at or near system II gives rise to the pale green condition of the mutants, this finding affords a rapid and convenient method for isolating strains of the organism which are unable to carry out normal photosynthesis.

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