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REVERSION OF *SCENEDESMUS* PHOTOSYNTHETIC MUTANTS

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SUMMARY

The chlorophyll–protein complex associated with Photosystem I, CP-I, which is absent in *Scenedesmus obliquus* mutant 8 grown in the dark, is present in light-grown photosynthetically competent cultures of *Scenedesmus* 8, contrary to a previously published report (Gee, R., Saltman, P. and Weaver, E. (1969) *Biochim. Biophys. Acta* 189, 106–115). This change from mutant to wild-type traits is not due to photoadaptation, but reflects the genetic reversion of some of the cells in the dark-grown population, as shown by the following evidence. (1) Individual cultures take different lengths of time to regain competence, whether started from the same or different dark-grown mutant 8 cultures. (2) Competent cells do not de-adapt when returned to the dark. (3) The appearance of wild-type traits is gradual. (4) A small number of cells in mutant populations are wild-type and are selected for in the light. The reversion rate of mutant 8 to the wild-type is high compared to that of mutant 11.

INTRODUCTION

The use of mutant strains of algae and higher plants has proven to be valuable in elucidating the numerous steps and components of the light reactions of photosynthesis. Gregory *et al.*[1] have recently shown that Bishop's *Scenedesmus obliquus* mutant 8 is missing the chlorophyll–protein complex associated with Photosystem I, and that the absence of this complex is probably responsible for the inability of the mutant to carry out photosynthesis. Gee *et al.*[2] have reported that mutant 8, when grown on the light for approx. 30 days, regains the ability to carry out photosynthesis. These investigators state that the mutant has the ability to photoadapt when grown in the light. It was, therefore, of interest to carry out experiments to determine whether the chlorophyll–protein complex, CP-I, re-appears during photoadaptation and, if this is so, to study the mechanism of its appearance. The experiments described in this paper show that the chlorophyll–protein complex, CP-I, does appear when mutant 8 is grown in the light and that the return to wild-type characteristics is associated with its presence. However, in the experiments reported here, this manifestation of wild-type traits is shown to be the result of a genetic reversion of some of the mutant 8 cells to wild-type. No photoadaptation of mutant 8 was observed.

Similar experiments utilizing mutant 11, a mutant which lacks Photosystem II

activity[3,4] were undertaken to ascertain whether it might also become photosynthetically competent when grown in the light. This strain was shown to retain its mutant traits when grown in the light to a much greater extent than does mutant 8. The reversion rate to wild-type of mutant 11 thus seems to be lower than that of mutant 8, perhaps because of the difference in the nature of the two mutations.

MATERIAL AND METHODS

Three strains of *Scenedesmus obliquus*, wild-type, mutant 8 and mutant 11, isolated by Dr. Norman Bishop[3,4] were cultured in the light or in the dark in a New Brunswick Psycro-Therm gyrotory shaker at 25 °C in the heterotrophic medium described by Bishop[4]. The minimal medium used was identical to Bishop's heterotrophic medium minus the glucose and yeast extract. The cultures were transferred every 7 days to fresh media.

The chlorophyll–protein complex associated with Photosystem I was prepared as described previously[1].

The wild-type strain and each of the mutants show a characteristic delayed light emission[5], and each culture was checked for its decay pattern before use. Delayed light measurements were carried out on a modified Becquerel phosphoroscope as described by Bertsch *et al.*[5] and the delayed light emitted from 1–3 ms after excitation was recorded. The ratio of the delayed light intensity at 1 ms after excitation to that at 3 ms is used as a convenient method for ascertaining whether the decay pattern is that of the wild-type strain, mutant 8, or mutant 11. The faster the decay, the larger the 1 ms/3 ms ratio. A ratio of above 3 is characteristic of a wild type decay, while a ratio below 1.8 is characteristic of mutant 8. Mutant 11 emits 250 times less delayed light than the wild-type strain.

$^{14}CO_2$ fixation experiments were carried out at 20 °C in a modified Warburg refrigerated water bath, with fluorescent lights under the bath illuminating the bottom of each flask with approx. $2 \cdot 10^4$ ergs/cm^2 per s. The organisms were harvested, washed twice in 0.05 M Tris buffer, pH 8.0, and added to 25-ml erlenmyer flasks at a concentration of 0.25 mg chlorophyll in 4.9 ml 0.05 M Tris buffer, pH 8.0. The cells, in buffer, were preincubated for 10 min in the light to eliminate residual CO_2 in the medium. They were then returned to the dark for 10 min to allow utilization of any high energy intermediates. In order to determine the dark $^{14}CO_2$ fixation, these cells were incubated in the dark for 20 min after the addition of 0.1 ml $^{14}CO_2$, in the form of 20 μmoles $NaH^{14}CO_3$ consisting of $7 \cdot 10^3$ cpm/μmole. 1-ml samples were removed and added to 4 ml boiling ethanol. The light was then turned on and the cells incubated for another 20 min in the light. 1-ml samples were again taken and added to 4 ml boiling ethanol. Aliquots of the killed cells in ethanol were plated and counted in a Nuclear Chicago low background gas flow counter after several drops of glacial acetic acid were added to remove any unassimilated $^{14}CO_2$. $NaH^{14}CO_3$ was purchased from New England Nuclear.

For oxygen evolution determinations, cultures were harvested, washed, suspended in minimal broth, and used directly at appropriate chlorophyll concentrations. Measurements were made with a Clark-type oxygen electrode at 25 °C using saturating white light.

Genetic reversion studies were carried out in the following manner. Dark-grown

mutant 8 cultures were harvested, washed twice with sterile minimal broth and re-suspended in sterile minimal broth. Equal aliquots were spread on minimal agar Petri plates. A small aliquot was spread on heterotrophic agar as a control. In some experiments, two aliquots equal to those spread on minimal agar were transferred asceptically to heterotrophic broth; one was grown in the dark and the other was grown in the light. Modifications are described in the results section. Similar experiments were carried out using mutant 11. As a control to check the media and the procedure, the wild-type strain, grown in the dark, was harvested, washed and resuspended in minimal broth and small aliquots spread on minimal agar. The mutants will not grow in minimal broth or on minimal agar in the light.

Chlorophyll determinations were carried out according to the method of MacKinney[6].

RESULTS AND DISCUSSION

In Table I, several characteristics of the *S. obliquus* mutant strains 8 and 11, grown in the light and in the dark, are compared to those of the wild-type strain grown in a similar manner. As previously reported, the chlorophyll–protein complex, CP-I, is missing in mutant 8 grown in the dark. The complex, however, is present in *Scenedesmus* 8 which has been grown in the light and has become wild-type in terms of photosynthetic ability. The presence of the CP-I complex, determined qualitatively appears when the population of mutant 8 cells has a detectable proportion of photosynthetically competent cells as measured by plate counts on minimal agar and by delayed light emission patterns. Mutant 8 cultures grown in the light which show no photosynthetically competent cells, even after growth in the light for several weeks, lack the CP-I complex. These observations confirm that the absence of the complex is responsible for the mutant traits of *S. obliquus* strain 8.

The results presented in Table I also show that wild-type cells grown in the dark

TABLE I

CHARACTERISTICS OF *SCENEDEMUS* STRAINS GROWN IN THE LIGHT AND IN THE DARK

Organism	*CP-I**	*$^{14}CO_2$ fixation*** (%)	*O_2 evolution**** (%)	*Delayed light (1 ms/3 ms)*
WT_{LT}	+	100	100	4.9
WT_{DK}	+	71	68	3.9
$Sc8_{LT}$§	+	91	103	4.1
$Sc8_{DK}$	—	0.9	-13	1.6
$Sc8_{LT-D_K}$§	+	66	63	3.6
$Sc11_{DK}$	+	2.2	0	Not measurable
$Sc11_{LT}$	+	—	0	Not measurable

* Indicates presence of CP-I on polyacrylamide gels (Gregory *et al.*[1]).

** 100% is equivalent to 9.7 μmoles $^{14}CO_2$/mg chlorophyll per h.

*** 100% is equivalent to 11.4 μmoles O_2/mg chlorophyll per h.

§ These organisms have reverted to the wild-type strain; refer to text for reversion time of each culture.

have a lower photosynthetic efficiency than those grown in the light. This is true for all of the photosynthetic parameters tested. Further studies on the differences in photosynthetic efficiency between light and dark-grown photosynthetically competent algal cells are under way.

Within 18 days after being placed in the light, the mutant 8 strain in Table I ($Sc8_{LT}$) became photosynthetically competent with regard to its ability to fix $^{14}CO_2$, evolve oxygen, and emit a wild-type delayed light decay. The $Sc8_{LT-DK}$ depicted in Table I represents a mutant which was wild-type by 35 days after initial exposure to light. An aliquot of the resultant "wild-type" culture was transferred to the dark and kept in the dark for 3 months (with transfers to fresh media every 7 days). The organism retained its wild-type characteristics. Its photosynthetic efficiency is comparable to that of the wild-type cells grown in the dark (WT_{DK}).

These results suggest that the conversion of mutant 8 to wild-type is not a photoadaptation but a genetic reversion. The hypothesis of photoadaptation leads to the prediction that all cells in the population should de-adapt when returned to the dark. Otherwise, one would never or rarely observe the photoadaptation phenomenon. All of the experiments carried out show that light-grown *Scenedesmus* 8 cultures, once having gained wild-type traits, retain these traits even when grown in the dark for long periods (the longest attempt was 8 months). The concept of photoadaptation also predicts that cultures started at different dates, under identical conditions, should photoadapt by the end of approximately the same time intervals. Of the two Sc8 cultures described in Table I, one became wild-type by 18 days and the other by 35 days after illumination. Other experiments were performed to check these observations. A number of cultures were transferred to fresh heterotrophic broth and grown in the light in a similar manner starting on different days. The conversion of mutant to wild-type traits were obtained by the end of vastly different time intervals, for example 16, 26, 28, 30 and 33 days.

Further studies were undertaken to test the photoadaptation hypothesis. Table II shows that three Sc8 cultures, started from the same dark-grown inoculum, have different rates of oxygen evolution and different delayed light decay patterns by the end of 28 days growth in the light. Thus, cultures started from the same

TABLE II

APPEARANCE OF WILD-TYPE TRAITS IN MUTANT 8 CULTURES

Three equal samples were taken from the same flask of Sc8 grown in the dark, inoculated into three different flasks of heterotrophic broth, grown in the light, and analyzed for wild-type characteristics after 28 days of growth. The wild-type strain grown in the light (WT_{LT}) and mutant 8 grown continuously in the dark ($Sc8_{DK}$) are presented as controls.

Organism	*O_2 evolution* (%)	*Delayed light* (*1 ms/3 ms*)
WT_{LT}	100	4.4
$Sc8_{LT-1}$	88	4.2
$Sc8_{LT-2}$	49	2.1
$Sc8_{LT-3}$	11	1.8
$Sc8_{DK}$	0	1.6

dark-grown inoculum assume wild-type traits at different times after initial exposure to light. These observations are in agreement with what one would expect if the phenomenon being studied were a genetic reversion.

The hypothesis of genetic reversion leads to the prediction that once wild-type traits appear, they are retained both in the light and in the dark. This was found to be the case. Also, the appearance of wild-type traits in liquid cultures should be gradual, reflecting a selection of one or more reverted cells. Fig. 1 shows the gradual conversion of the delayed light decay pattern from the mutant to the wild-type decay of a culture inoculated with dark-grown mutant 8 and maintained in the light for the indicated number of days.

Several types of experiments were undertaken to verify the aforementioned observations favoring the genetic reversion hypothesis. One of these involved determining whether a mutant population had any cells that could grow in liquid minimal media. Dark-grown mutant 8 cultures, harvested, washed and inoculated into flasks of minimal broth, gave rise to small populations of cells that showed wild-type delayed light decay curves after several weeks of incubation in the light. This period of time was probably necessary for the one or more wild-type cells in the mutant population to grow up to an observable number.

Finally, studies were undertaken which conclusively show that only some cells in a mutant 8 dark-grown population are wild-type and that these cells are selected for in the light. Mutant 8 dark-grown cultures were harvested, washed and suspended in minimal broth as described in the methods. The resulting suspension contained at least 10^9 cells. All of these cells were spread on minimal agar Petri plates, with approx.

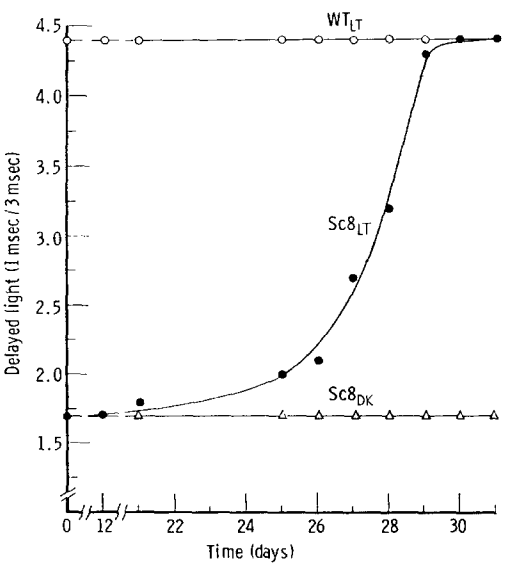

Fig. 1. The gradual appearance of wild-type delayed light characteristics by *S. obliquus* mutant 8 grown in the light. All cultures were grown in heterotrophic medium as described in the text. WT$_{LT}$, wild-type strain grown continuously in the light. Sc8$_{DK}$, mutant 8 strain grown continuously in the dark. Sc8$_{LT}$, mutant 8 strain which has been transferred from the dark and grown in the light for 31 days.

10^8 cells per plate. A total of one to three colonies grew on all of the plates inoculated, in several different experiments. These colonies were shown to be wild-type by re-streaking them on minimal agar and obtaining abundant growth. Such results imply that 1, 2 or 3 cells in a population of approx. 10^9 cells show wild-type traits in a dark-grown culture. Wild-type control minimal agar Petri plates were over-grown when spread with many fewer organisms. No colonies have so far appeared on Petri plates in similar experiments with mutant 11.

For some experiments, in addition to spreading dark-grown cells on minimal agar, a 10^8-cell sample was placed in heterotrophic broth and grown in the dark while an equivalent number of cells were added to heterotrophic broth and grown in the light. After one week of growth, 10^8-cell aliquots were removed from each of the flasks, washed, suspended in minimal broth and spread on minimal agar. In one such experiment, two colonies appeared on the Petri plates spread with the dark-grown cultures whereas a number of colonies representing at least 340 cells in the light-grown culture were present on the plates. After another week of growth, 10^8-cell aliquots were again removed from each of the flasks and treated as above. Many colonies, at least 3000 per plate, were present in the light grown culture, representing at least $4 \cdot 10^5$ cells. No colonies were present on the Petri plates spread with an equivalent aliquot of the dark-grown mutant 8 cells. These results unambiguously show that mutant 8 populations (of at least 10^9 cells) tend to have one or more cells which have reverted to wild-type, that these cells have a selective advantage when grown in the light, and that this selective advantage results in the gradual appearance of wild-type traits in a population of mutant cells grown in the light. There is no selective advantage in the dark for the revertant. The predominant appearance of wild-type traits at different times in different cultures reflects the number of reverted cells present in the original culture or the spontaneous appearance of revertants while the cells are growing in the light.

The reversion rate of mutant 11 to wild-type appears to be relatively low compared to that for mutant 8. In only one out of 10 experiments has a liquid culture of mutant 11 growing in the light shown wild-type traits (delayed light emission and oxygen evolution).

Obviously, a study of the chlorophyll–protein complex, CP-I, in mutant 8 is still of great interest. The high reversion rate might be a reflection of the type of mutation. It is probably not a deletion, but is more likely a single base change which may result in a nonsense mutation or in the substitution of one amino acid for another. Further studies along this line might shed some light on the properties of CP-I. Questions remaining to be answered include whether the protein subunits are missing in the mutant, or if present, whether they are unable to agregate with each other and/or with chlorophyll to form a functional chlorophyll–protein complex.

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