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PHOTOBLEACHING AND DARK-BLEACHING OF EUGLENA GRACILIS CHLOROPLAST FRAGMENTS

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SUMMARY

Photobleaching and dark-bleaching of *Euglena gracilis* chloroplast fragments were studied at different pH. Moreover, the effect of these processes on low-temperature fluorescence was investigated.

The photobleaching of the long-wave chlorophyll *a* forms C_a704 and C_a692 proceeded at a higher rate than that of the C_a681, C_a672, and C_a664 forms. Chlorophyll *b* is considerably more stable towards this process than the C_a components. At pH 7.10 the absorption around 649 nm even increased. The percentual quenching of fluorescence was much higher than the absorbance decreases.

The dark-bleaching patterns of the C_a704 and C_a692 forms showed mutual differences. Moreover, they differed from those of the remaining C_a forms. Dark-bleaching of chlorophyll *b* did not notably diverge from that of the latter C_a forms. The percentual fluorescence decline was much higher than the decreases of absorbance also here.

The results are discussed.

1. INTRODUCTION

In the *in vivo* state, chlorophylls are irreversibly bleached by exposure to high light intensities (THOMAS & NIJHUIS 1968) as well as prolonged incubation in darkness (BROWN 1963). The various forms of chlorophyll *a* (FRENCH et al. 1972; BROWN 1972) as well as the chlorophyll *b* complex bleach at different rates (THOMAS & NIJHUIS 1968; BROWN 1963).

The mechanisms differ for bleaching by light and in darkness. The former process regards a cyclic peroxidation resulting in bleaching of endogenous chlorophyll, production of malondialdehyde due to destruction of three-fold unsaturated fatty acids, and oxygen uptake (HEATH & PACKER 1968). It is suggested that the very first step of this process consists of a 1,4-peroxidation of chlorophyll (SHERMAN et al. 1972). More details about the various processes are presented in literature, e.g. HARNISCHFEGER (1972) and HOSHINA et al. (1975).

Dark-bleaching in intact cells is not comparable to dark-bleaching of isolated chloroplasts (WINTERMANS 1967). In intact cells it is likely to be initiated by hydrolytic enzymes located in the cytoplasm. Therefore, dark-bleaching is considerably retarded by isolating the chloroplasts (MARTIN & THIMANN 1972;

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CHOE & THIMANN 1974, 1975). Polyphenol oxidase activity together with the presence of linolenic acid seems to be one of the factors responsible for the ageing of isolated chloroplasts (SIEGENTHALER 1970).

Since various chlorophyll forms show a mutually different bleaching pattern, comparison of bleaching characteristics in light and in darkness may yield some information about differences in the nature of these chlorophyll complexes. As, most likely, lipoproteins function as carriers of this pigment *in vivo* it seemed worthwhile to examine the bleaching phenomena at various pH's. As both kinds of bleaching processes occur at a relatively high rate in *Euglena gracilis*, this organism is used in the present investigation. The results of this study are presented below.

2. MATERIAL AND METHODS

Preparation: Euglena gracilis was grown in a 0.5% solution of Difco Bacto peptone in tapwater. The culture flasks containing 25 or 50 ml of the medium were placed in a Psycrotherm incubator shaker under air enriched with 5% CO_2 at 27 °C. Throughout the growing period of 7 days a light-dark cycle of 14 and 10 hours respectively was maintained.

The cells were spun down at about 2500 × *g* for 10 min. Next, the sediment was taken up in a few ml of 0.02 M citrate solution, pH 7.0, and upon addition of carborundum "F" the mixture was thouroughly ground in an agate mortar for 10 min. Thereupon the cell debris and the carborundum were removed by three-fold centrifugation at 150 × *g* for 5 min., at 1200 × *g* for 10 min., and at 1500 × *g* for 10 min. respectively. The final supernate, containing the chloroplast fragments, was centrifuged at 10.000 × *g* for 20 min. at 0 °C. The resulting sediment was suspended in a mixture of 2 parts reagent grade glycerol and 1 part citrate solution of the desired pH, up to a final concentration of 0.02 M. Experiments were performed at pH 5.15, 7.10, and 8.85 respectively.

Absorption measurements: Absorption spectra were recorded in a Cary Model 14R spectrophotometer at both room and liquid nitrogen temperature. Perspex cuvettes, light path 1 mm, were used.

Fluorescence measurements: Emission spectra were established with the apparatus described by GOEDHEER (1964) at both mentioned temperatures. The preparations were placed into a 1 mm perspex cuvette as used by BRIL et al. (1969). The geometry of the experimental set-up was checked by means of a fluorescein sample.

Data processing: The absorption spectra were analyzed by computer with the aid of the RESOLV program developed by Dr. D. D. Tunnicliff of the Shell Development Company, Houston, Texas. A revised version, kindly provided by Dr. C. S. French was used after some minor changes due to the type of

computer, cf. OUDSHOORN & THOMAS (1975). The simple component was chosen as a mixture of a Gaussian and a Lorentzian curve.

Difference spectra were established by the computer as well. The pertaining program was developed at our institute by Mr. J. W. Kleinen Hammans.

Bleaching procedures: The samples were pipetted into 1 cm glass cuvettes. Sedimentation of the suspension was prevented by the use of an electromagnetic stirrer throughout the photobleaching experiments. In case of the long-term experiments on dark-bleaching this stirrer was operated prior to each set of measurements for 30 min.

The intensity of the incandescent light used for photobleaching amounted to 0.09 $W.m^{-2}$ at the front wall of the cuvette. The maximum absorbance of the suspension was adjusted to about 0.7 in a 1 mm cuvette at 77 °K. During irradiation the temperature was kept constant at 20 °C.

Dark-bleaching was performed at the same temperature, except for two experiments at pH 7.10 and one experiment at pH 5.15, where the temperature fluctuated in between 20 and 21 °C. The results, however, were not markedly different. The maximum absorbance under the mentioned conditions ranged in between 0.4 and 0.6.

3. RESULTS

Curve analyses of low-temperature absorption spectra in the 640–720 nm region yielded components peaking at 701.–708. nm, 691.3–692.8 nm, 680.6–681.6 nm, 671.8–672.5 nm, 663.9–664.1 nm, and 649.3–649.7 nm respectively. Using the same sequence, these components will be termed below: C_a704, C_a692, C_a681, C_a672, C_a664, and C_b649. The latter component represents chlorophyll *b*, whereas the other ones refer to chlorophyll *a* forms. An example is shown in *fig. 1*.

Comparison of low-temperature absorption spectra of intact cell and chloroplast fragment suspensions from the same culture showed that the preparation procedure caused some minor absorption changes. In a single experiment the fragment samples showed a relative increase of the C_a672 component, amounting to 4.7%, as well as a relative decrease of 5.4% for the C_a692 complex. The low-temperature emission spectra differed as well. The fluorescence maximum for intact cells and chloroplast fragments was located at 721 nm and 725 nm respectively. Due to a reduction of the emission at the short-wave side of the complex band in fragment suspensions, the halfwidth values for intact cells and chloroplast fragments amounted to 50 nm and 39 nm respectively.

Glycerol in a concentration as added to the samples in order to enable the recording of low-temperature spectra prevented bacterial growth in long-term experiments, but it reduced the bleaching rates. Comparison of room-temperature spectra in a single experiment showed that photobleaching of glycerol-containing samples was 10–15% less than that of glycerol-free ones. At an

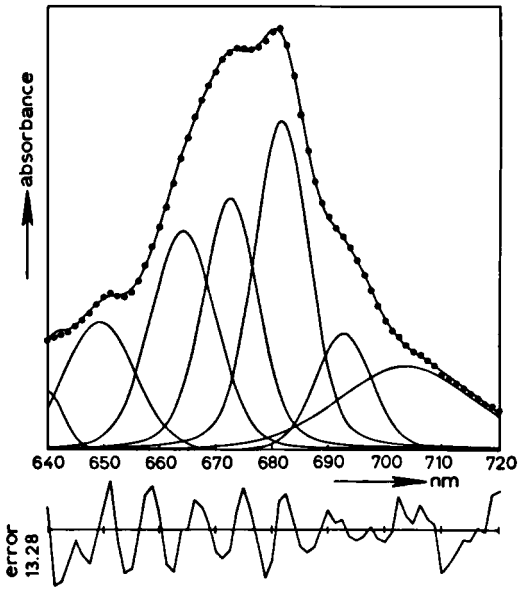

Fig. 1. Example of the used curve analysis.

incubation period of 6 h. and at 20°C this percentage amounted to about 5% for darkbleaching. In both types of bleaching, the C_a672 component was less affected by the presence of glycerol than other chlorophyll forms, whereas this chemical caused a relative protection of C_a704 with regard to photobleaching, but a decreased resistance towards dark-bleaching of the latter component.

Photobleaching

Effects on absorbance. The proceeding of photobleaching is shown in *fig. 2A* for the various components. The values represent means of 3 experiments. Though the bleaching rates of samples from each series may somewhat differ, the bleaching pattern of the individual experiments is of the same type. The data from *fig. 2A* show that the bleaching rates of the long-wave C_a forms C_a704 and C_a692 are higher than those of the forms absorbing at shorter wavelengths: C_a681, C_a672 and C_a664. Chlorophyll *b* is much more stable towards photobleaching than the C_a forms at low and high pH. At pH 7.10 the absorption around 649 nm shows an increase instead of a decrease. As a rule the photobleaching of the various components slightly decreases with lowering of the pH, cf. also *fig. 3.*

The C_a664 photobleaching is somewhat decreased at pH 7.10 when compared to that at high and low pH. In one of the experiments from this series a slight absorption increase occurred upon 15 and 45 min. of irradiation at the neutral pH.

Effects on fluorescence. As it is shown in *figs. 2A* and *3*, the lowtemperature fluorescence is readily quenched due to photobleaching. The effect of pH on this phenomenon is only rather poor.

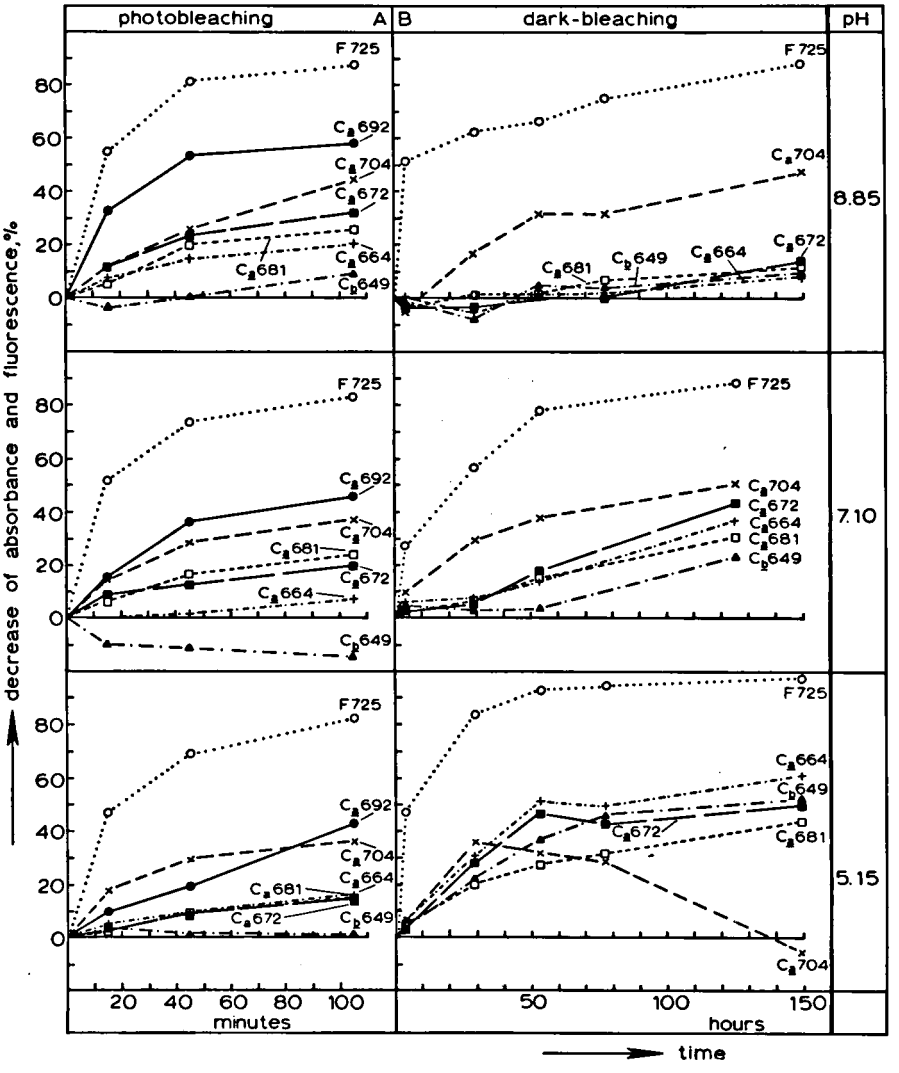

Fig. 2. Effect of bleaching at different pH on absorbance of the diverse chlorophyll components and fluorescence at 77 °K. C_a, chlorophyll *a* forms with maximum absorbance at the indicated wavelenghts; C_b649, chlorophyll *b*; F725, fluorescence band around 725 nm. A, photobleaching; B, dark-bleaching. Except for the data referring to dark-bleaching at pH 7.10 for 4h., as well as at pH 5.15 for 77h. and 149h., which are means for only two experiments, the values represent means of three experiments.

Dark-bleaching

Effects on absorbance. The time course of dark-bleaching is presented in *fig. 2B* for the various components and at different pH. The values represent means of three experiments. C_a704 bleaches considerably faster than the other forms at high pH. At pH 7.10 this difference is reduced, whereas at low pH a slowing down of the bleaching rate occurs after 29 h. As to the other C_a forms, except for C_a692 as mentioned below, as well as C_b, *cf.* also *fig. 3*, the bleaching rates of these components do not considerably diverge from each other. In contrast to photobleaching, dark-bleaching is highest at low pH.

The dark-bleaching pattern of C_a692 proved to be quite irreproducible. Therefore, the pertaining data of this pigment form are not shown in *figs. 2* and *3*, but the results of the individual experiments are presented in *fig. 4*. A possible reason for the divergence of these data will be considered in the discussion.

Effects on fluorescence. Figs. 2B and *3* demonstrate that, due to dark-bleaching, the fluorescence at 77°K decreased considerably faster than the absorbance of the various chlorophyll components. The fluorescence quenching proceeded at a higher rate when lowering the pH. A 90% reduction was observed after an incubation of 50 h. at pH 5.15, whereas this percentage was reached after 125 h. and 150 h. at pH 7.10 and 8.85 respectively.

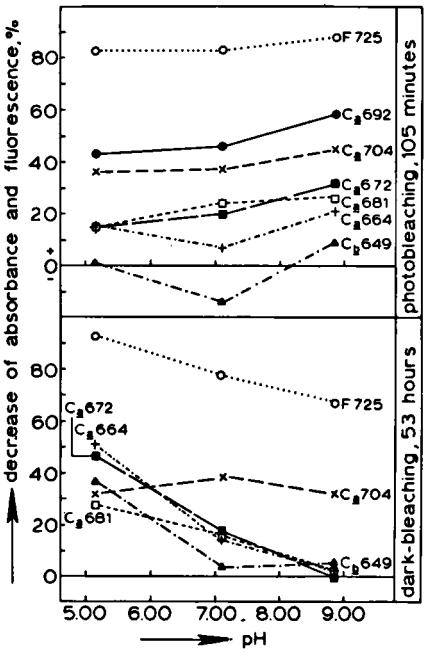

Fig. 3. Comparison of the effect of pH on photobleaching and dark-bleaching of about the same extent. For symbols see legends of *fig. 2*.

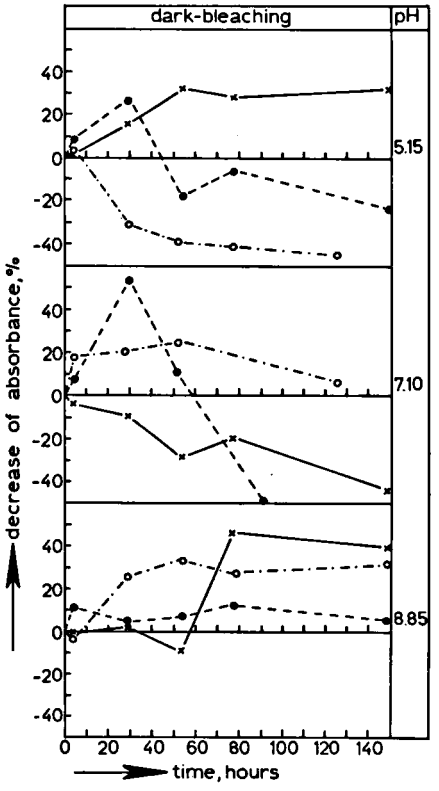

Fig. 4. Effect of dark-bleaching at different pH on absorbance of C_a692 at 77°K. In order to demonstrate the irreproducibility of the bleaching patterns of this particular pigment form, the data refer to single experiments.

4. DISCUSSION

According to the photobleaching rates, two classes of C_a forms can be distinguished. The long-wave forms C_a704 and C_a692 show a higher bleaching rate than the shorter-wave components C_a681, C_a672, and C_a664. Though this holds for the photobleaching at the three studied pH's, it can be most clearly observed at low pH, cf. *fig. 2A*. A possible explanation for the higher bleaching rates of the C_a704 and C_a692 forms may be that, due to energy transfer from the shorter-wave forms, the excitation of the long-wave components is increased, and, with it, the destructive oxidation.

The photobleaching of C_b differs considerably from that of the C_a forms. At both high and low pH the bleaching is only weak. At pH 8.85, instead of a decrease, a slight increase of absorbance was observed upon a 15 minutes' irradiation. At pH 7.10 a clear increase of absorbance occurred throughout the

105 minutes' period of photobleaching. Since this process is of an oxidative character, one may wonder whether at neutral pH, and also in the early stages of photobleaching at pH 8.85, the methyl group at the C-3 position is oxidized to a carbonyl group, in this way forming C_b from C_a. Further study is required to clarify this problem.

The decrease in the low-temperature fluorescence is considerably higher than that of the absorbance of the chlorophyll components, whereas its sensitivity towards pH is rather low.

As mentioned in the Introduction, other enzymes are involved in dark-bleaching than in photobleaching. It is obvious from *fig. 2B* that the C_a704 dark-bleaching pattern differs from that of the other C_a forms. These results may suggest that the lipoprotein carrier of C_a704 differs from those of the remaining C_a complexes. The carriers from the latter forms may be mutually different as well. However, the dark-bleaching patterns of these components differ too little to justify such a conclusion. The shorter-wave C_a forms and C_b show about the same pattern of dark-bleaching. The irreproducibility of the C_a692 dark-bleaching pattern might be due to a location of this form at the surface of the membrane. For, if so, C_a692 is readily accessible for the oxidizing enzymes from the cytoplasm, whereas in the various preparations the concentrations of the enzymes may differ.

Incubation in darkness also reduces the low-temperature fluorescence considerably more than the absorbance of the various chlorophyll components. The fluorescence quenching at pH 8.85 in particular suggests a biphasic nature of this process. Future research is needed to obtain more insight into this matter.

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