LIGHT AND HEAT IN THE BLEACHING OF CHLOROPLASTIN *EUGLENA**

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We have been studying the effects of temperature, light intensity and wavelength on the structure of the chloroplast, and on its pigments, chlorophyll and carotenoids, in the algal flagellate, *Euglena gracilis*[1,2,3,4]. For *Euglena* in the light, the rate of chlorophyll synthesis increases with temperature, but degradation of chlorophyll becomes noticeable at 43° C within an hour, and at 48° C within 30 minutes[3]. In darkness chlorophyll is bleached at a rate which is independent of the experimental temperature below 32° C, but at a rate which is temperature-dependent above 32° C[5]. Although it is difficult to study quantitatively the degradation of chlorophyll within the organism[6], some data were obtained for the rate of "bleaching" in the first few hours at 38–48° C in white light. It was also noted that the bleaching of chlorophyll within the organism takes place more rapidly in red light.

The pigment–macromolecule complex, chloroplastin**, can be extracted from *Euglena* chloroplasts by detergents[7,8]. The absorption maxima of this preparation coincides with those of the living cell (Fig. 1). *Euglena* chloroplastin sediments in the analytical ultracentrifuge as a single component. We have calculated from the sedimentation constant, percentage nitrogen, and dry-weight of the complex, that one pigment molecule of chlorophyll is most probably associated with one macromolecule. The calculated average molecular weight of the complex, from its sedimentation constant in the analytical ultracentrifuge and its percentage nitrogen, is 37,000[9,10].

In the present research chloroplastin was used to see whether kinetic studies would be helpful in learning more about the "bleaching" of chlorophyll *in vivo* and to study the relation between light and heat bleaching in the pigment-complex.

EXPERIMENTAL METHODS

Euglena gracilis var. *bacillaris* was used throughout these studies. All studies were performed using a Bronwill temperature-controlled (± 0.1° C) glass water bath with a 40 W circular G.E. fluorescent tube as a light source.

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** "Chloroplastin", as defined here, appears to be similar to SMITH AND YOUNG's *chlorophyll holochrome*[11], chlorophyll in its natural state, combined with proteins, carotenoids, lipids and lipoproteins.

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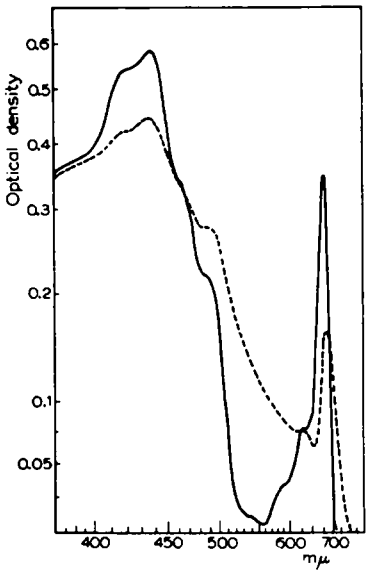

Fig. 1. A comparison of the absorption spectra of 1.8% digitonin extract of chloroplastin and a suspension of *Euglena gracilis*. Recordings with Beckman DK Spectrophotometer. ——— Chloroplastin (digitonin complex); ----- *in vivo Euglena g.*

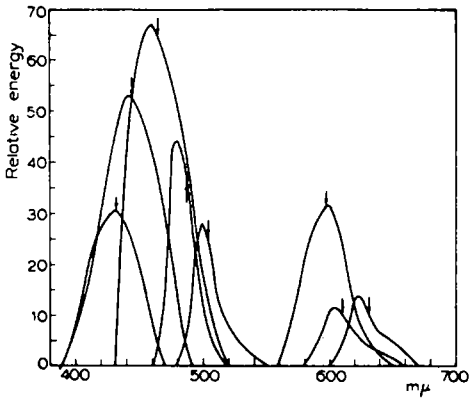

Fig. 2. Effective energy distribution of various filter combinations, obtained by multiplying together (1) the relative energy of the filters, (2) the energy distribution of the light source, and (3) the absorption spectrum of chloroplastin (as relative absorbed energy). The arrows denote the dominant wavelength of each filter combination. The filters are listed in Table I.

Chloroplastin was prepared from light-grown cultures of *Euglena* in the logarithmic phase of growth, in a manner similar to that of SMITH[7,8]. Freshly harvested cells were washed with distilled water and frozen. The pellet was permitted to thaw and then ground vigorously for 15 minutes with clean white sand. The freezing and thawing aids in the disruption of the cellular membrane. The ground material was suspended in water and centrifuged at low speed (1800 r.p.m.) for 10 minutes to get rid of sand and cellular debris. The supernatant was recentrifuged at higher speed (3500 r.p.m.) for 10 minutes. The material remaining in suspension was mostly chloroplasts and some intact organisms. This was centrifuged at 1200 r.p.m. for 15 minutes and the pellet, containing mostly chloroplasts, was extracted for 1–4 hours in darkness at room temperature, with 1.8% aqueous digitonin* buffered with sodium borate. The extract was centrifuged at 12,000 r.p.m. for 15 minutes to obtain a clear solution. The pH of the final extract was 7.2. The dry weight of the complex averaged 28.63 mg/ml, of which $6.06 \cdot 10^{-5}$ moles/liter was chlorophyll and 0.36 mg/ml nitrogen by analysis[9,10].

100-ml Petroff flasks were used as the experimental reaction vessels. The entire surface of each flask was painted with black enamel, except for a 16 cm^2 area window on the front, over which the various light filter combinations were placed. The relative energy distribution of the light source and of each filter combination was obtained from Beckman DK Spectrophotometer recordings in a manner previously described[3,4]. The relative energy of the filters, the energy distribution of the lamp, and the absorption spectrum of chloroplastin (as relative absorbed energy rather than optical density) were multiplied together at appropriate wavelengths, and the resulting energy values were plotted against wavelength, for each filter combination. The wavelength which corresponded to the center of gravity for the energy distribution curve of each light filter combination was taken as the dominant wavelength of that combination. Filter energy distribution curves are shown in Fig. 2. The filters used are listed in Table I.

The experimental vessels were flushed with nitrogen before introducing the pigment complex. 50 ml of chloroplastin, (the pigment-macromolecular complex) were placed in each flask, and the flasks again filled with nitrogen to prevent photooxidation. The flasks were suspended around the circumference of the bath.

Bleaching was measured in terms of decrease in optical density or increase in per cent transmission of chloroplastin with time at 675 mμ, as recorded with a Beckman DK spectrophotometer.

* Digitonin—$C_{55}H_{90}O_{29}$ anionic reagent, D_{58}, Fisher Scientific Company.

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TABLE I

FILTER COMBINATIONS

Filter combinations	*Center of gravity of the transmission band*	*% Relative energy transmitted at center of gravity of transmission band*
Corning 5850 + 5543*	432 mμ	8.0
Corning 9780 (½ thickness) + 5543*	444 mμ	9.0
Corning 3389 + 5562*	465 mμ	10.0
Corning 3385 + 5030*	487 mμ	8.0
Corning 3384 + 9780*	504 mμ	9.0
Corning 3384 + 3480*	596 mμ	8.0
Corning 2424 + 2434	610 mμ	8.5
Corning 2412	630 mμ	8.5

* Neutral filters were used to reduce the light intensity.

EXPERIMENTAL DATA

I. Chlorophyll in vivo

A. *Temperature dependence of bleaching.* Previously, we have studied the irreversible *in vivo* temperature bleaching of *Euglena* at 33°, 38°, 40°, 43°, and 48° C[3]. The pH of the culture medium varied from 7–8 on harvesting the cells. The absorption spectra of acetone extracts (pH 7.0) of the bleached cells showed a decrease in the pigment concentration, and a replacement of the major chlorophyll absorption peaks by those of pheophytin.

These measurements of chlorophyll concentration calculated from the absorption spectra are used in Fig. 3 to construct a plot of the log of the concentration (in

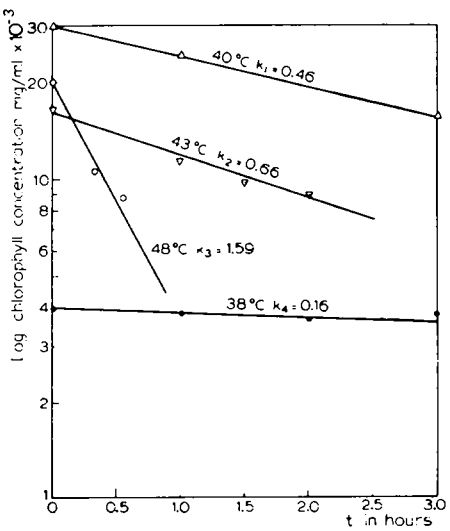

Fig. 3. The bleaching of chlorophyll in *Euglena* in white light at various temperatures. The slopes of these lines, k_1, k_2, k_3, and k_4, are the reaction rate constants in reciprocal hours for each temperature.

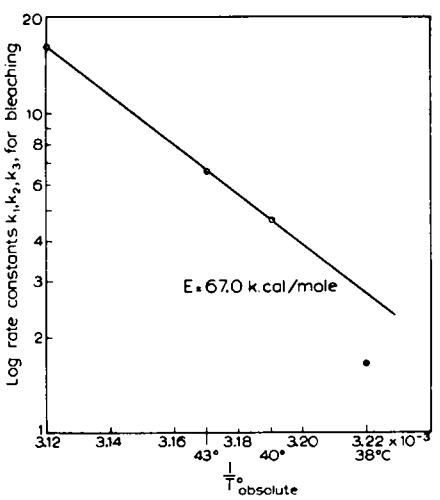

Fig. 4. The log rate constants for bleaching of chlorophyll in *Euglena* in white light plotted against the inverse of the absolute temperature. From the slope of this line, an activation energy for bleaching of 67.0 kcal/mole has been calculated.

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mg/liter) at various temperatures as a function of time. At a temperature (38° C) close to the optimum for cellular growth, the concentration of the pigment remains almost constant for the experimental time. At higher temperatures, bleaching occurs coupled with general deterioration and ultimate death of the cells. The slopes of the lines in Fig. 3, k_1, k_2, k_3, and k_4 represent reaction rate constants in reciprocal hours. If they are plotted against the reciprocal of absolute temperature, a straight line is obtained (Fig. 4). From the slope of the curve in Fig. 4, the activation energy of bleaching of cells was calculated, using the three available combinations of k values (k_1, k_2, and k_3). A mean value of 67 kcal/mole was obtained.

II. Chloroplastin

A. *Bleaching in white light and darkness.* In white light, the bleaching of chloroplastin was observed at 10° C, 25° C, 30° C and 40° C; this rate is practically independent of temperature (Fig. 6a). The absorption spectra of the bleached chloroplastin preparations are illustrated in Fig. 5. They too, are the same for all temperatures studied.

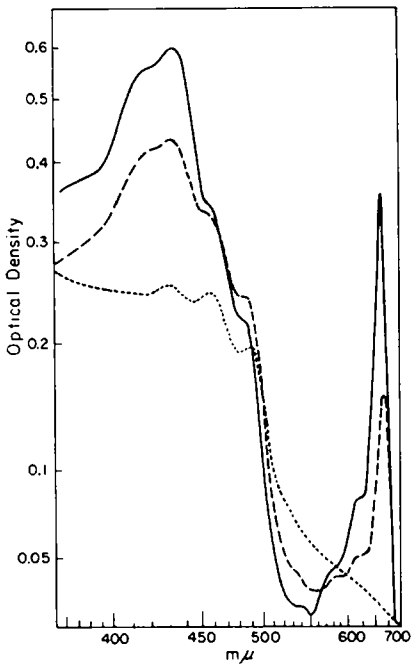

Fig. 5. Bleaching of chloroplastin in white light at 25° C. Extract prepared from light-grown *Euglena* in 1.8% digitonin, pH 7.2. ——— o hours; – – – – 1.5 hours; ····· 24 hours, 25° C in white light.

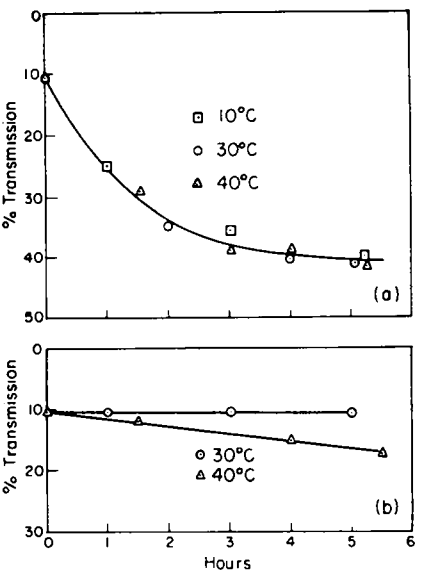

Fig. 6. (a) Chloroplastin bleaching in white light at various temperatures. (b) Thermal bleaching of chloroplastin in darkness. Bleaching measured as increase in % transmission, at 675 mμ, of the chloroplastin (pH 7.2) with time.

In darkness at 30° C, the increase in transmission of the chloroplastin preparation is less than 6% in 24 hours, but bleaching is rapid when the temperature is raised to 40° C (Fig. 6b). The ratio between the rates of bleaching at 30° C and 40° C or Q_{10}, is 13.25. The activation energy of the thermal bleaching of chloroplastin, calculated by applying Arrhenius' equation:

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$$\frac{\mathrm{d} \ln k}{\mathrm{d}T} = \frac{E}{RT^2} \qquad (1)$$

where k is the rate constant, T the absolute temperature, E the activation energy, R the gas constant. The equation was used in the form:

$$\ln \frac{k_2}{k_1} = \frac{E}{R} \frac{T_2 - T_1}{T_1 T_2}$$

The calculated value for these two rates is 48.2 kcal/mole.

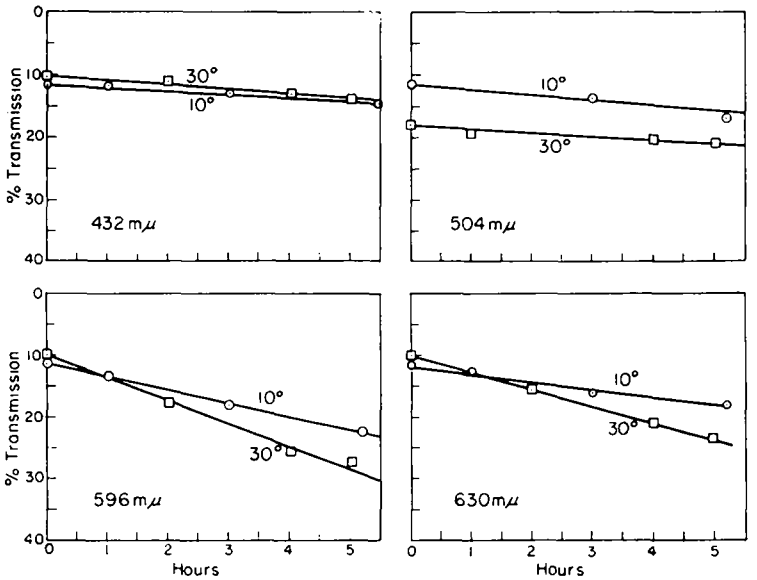

Fig. 7. Typical curves showing bleaching rates of chloroplastin at various wavelengths. No temperature-dependence is found in the bleaching rate below 504 mμ, but high temperature-dependence can be noted at 596 mμ and 630 mμ.

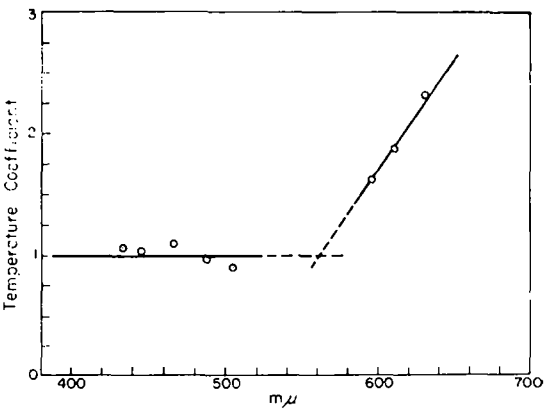

Fig. 8. The temperature dependence of the bleaching rate of chloroplastin expressed as a function of wavelength. The intersection of the straight lines drawn through the experimental points indicates that temperature-dependence begins at about 560 mμ.

B. *The temperature dependence of bleaching in "monochromatic" light.* The chloroplastin was bleached at various wavelengths at 10° C and 30° C. Typical bleaching curves are shown in Fig. 7. It can be seen that the k_2/k_1 ratio is ~ 1 at 504 mμ and slightly < 1 at 432 mμ. At 596 and 630 mμ however, the bleaching rate is higher at the higher temperature. Fig. 8 shows the temperature dependence of the rate as a function of wavelength. (Owing to the low absorption of chloroplastin at 520–580 mμ, no

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measurements could be made in that region.) Bleaching becomes temperature-dependent at about 560 mμ (Fig. 8). The quantum energy at 560 mμ corresponds to about 48.3 kcal, which is equal to the activation energy calculated above for thermal bleaching in darkness.

The thermal dependence of bleaching increases with wavelength (Table II). The total activation energy is the sum of the quantum energy and the thermal activation energy (as calculated from the Arrhenius equation). This sum is found to remain constant from 560 mμ into the red (Table II).

TABLE II

ACTIVATION ENERGY OF CHLOROPLASTIN BLEACHING

Wavelength mμ	Q_{20}	*Arrhenius energy* kcal/mole	*Quantum energy* kcal/mole	*Total energy* kcal/mole
Darkness	—	48.0	—	48.0
504	0.67	−3.36	52.8	49.4
596	1.46	3.26	44.6	47.9
610	1.54	3.73	43.6	47.3
630	2.01	6.02	42.3	48.1

DISCUSSION

In *Euglena* chlorophyll can be bleached by darkness alone, and in light or darkness at temperatures above 32° C. The bleaching of chlorophyll was measured by the increase in percentage transmission or decrease in optical density with the disappearance of the red absorption band at 675 mμ (Fig. 5).

Studies of chlorophyll bleaching in alcohol or acetone solutions cannot be easily related to the reactions in photosynthesis since it is necessary to imitate more closely the conditions in the cells[14]. *Chloroplastin*, the extractable pigment–macromolecular complex in digitonin, is similar in its absorption spectra to the organisms *in vivo* (Fig. 1), and it could photocatalyze and reduce the dye 2,6-dichlorobenzenone indophenol[13]. The bleaching as observed here is not due to photooxidation, since the chloroplastin is in an atmosphere of nitrogen.

The bleaching of chloroplastin can be produced by light, by heat, or a combination of both. Below 560 mμ bleaching is accomplished by light energy alone; above 560 mμ it requires the combined effects of light and heat. The total activation energy of bleaching is the same in darkness and in light 48.3 kcal/mole.

A value of 67 kcal/mole can be calculated for the bleaching *in vivo*—somewhat higher than for the chloroplastin extract. Both values fall within the range of values which have been found for denaturation of proteins[14,15].

Since the function of photoreceptors (photosynthetic or visual) is to "trap" light energy for transfer to chemical or electrical energy, and since there is a similarity in structure of the photoreceptors as well as the pigment complex in digitonin (as indicated by the electron micrographs and the sedimentation in the analytical ultracentrifuge), it is interesting to compare the light and heat bleaching of chloroplastin to that of the visual pigment complex, rhodopsin, as studied by ST. GEORGE[16]. Recently, it has been demonstrated that chloroplasts, as well as retinal rods and cones, are composed of lamellar plates or discs 100 A in thickness[9,10,17,18]. A homo-

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geneous complex can be extracted from both these photoreceptors with digitonin, containing one pigment molecule per macromolecule, and having a molecular weight of 37,000 for *Euglena* chloroplastin and 40,000 for cattle rhodopsin[19], as determined by its sedimentation constant in the analytical ultracentrifuge and percentage nitrogen in the complex.

Rhodopsin has been shown to bleach by both light and heat[16, 20]. LYTHGOE AND QUILLIAM[20] found that rhodopsin bleaches rapidly in the dark at temperatures of 50° C and above, and have calculated an activation energy of 44 kcal/mole for its bleaching in neutral solutions. The combined effects of light and heat as observed in the bleaching at any temperature in white light is nearly unity for rhodopsin[21]. ST. GEORGE[16] showed that the bleaching of rhodopsin in light becomes temperature-dependent at about 590 mμ. He calculated an activation energy of 48.5 kcal/mole for bleaching in light. When the quantum energy decreases below 48.5 kcal/mole—the energy of the quantum at 590 mμ—the temperature coefficient increases in the same way as we noted for chloroplastin above 560 mμ. We are not implying that photosynthesis and vision are the same, but only noting a comparative similarity for the minimum experimental activation energies for bleaching *Euglena* chloroplastin to that of frog and cattle rhodopsin, as given in Table III.

TABLE III

	Comparative rate of bleaching at all temperatures in white light	*Calculated experimental activation for bleaching in darkness*	*Experimental wavelength at which temperature dependence of bleaching begins*	*Calculated experimental activation energy for bleaching in light*
Chloroplastin *Euglena g.*	1	48.2 kcal/mole	560 mμ	48.3 kcal/mole
Rhodopsin[16] frog, cattle	1	44.0 kcal/mole[20]	590 mμ	48.5 kcal/mole

Energy transference between the prosthetic group and the protein is discussed by ST. GEORGE[16] in connection with rhodopsin. He indicates from the work of DUTTON, MANNING AND DUGGAR[22], that the protein of the photosynthetic complex is involved in the transference of energy. DUYSENS indicates that the transference of energy can come from other pigments (carotenoids) to chlorophyll[12]. To understand the complete process of bleaching, it is necessary to understand the mechanisms involved. From what we have studied, it appears that there would probably be more than one independent mechanism involved in transferring and transforming the light and heat energy.

SUMMARY

1. Chlorophyll in *Euglena* can be bleached by darkness as well as in light at temperatures above 32° C with an activation energy of 67 kcal/mole.

2. (a) Chloroplastin can be bleached by light, heat, and a combination of both light and heat. (b) The rate of bleaching of chloroplastin in white light is independent of temperature; the rate of bleaching in darkness becomes temperature-dependent above 30° C. (c) There is a critical wave length (560 mμ) beyond which bleaching by light becomes temperature-dependent. (d) The total energy needed to bleach chloroplastin in light and in darkness is a constant, 48.3 kcal/mole.

3. The light and heat bleaching of chloroplastin, the photosynthetic complex, has some similarities to that of rhodopsin, the visual pigment complex.

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SOME CYTOCHEMICAL CHARACTERISTICS OF A PHOSPHORYLATING DIGITONIN PREPARATION OF MITOCHONDRIA

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The mechanism and even the constituents of the oxidative phosphorylating system of mitochondria are largely unknown. Earlier work[1] has given indications that mitochondrially-bound nucleotides, mostly of the adenine type, are involved in this process, since when mitochondria are treated so that they lose their phosphorylating ability, they also lose their bound nucleotides. It has recently been shown[2,3,4] that extracts of mammalian mitochondria could be prepared which contained the enzymes necessary for oxidative phosphorylation, but there was no indication of the presence or absence of possible co-factors such as the intra-mitochondrial nucleotides. This paper describes the properties of a digitonin preparation of broken mitochondria, and in brief it shows that this preparation contains no whole mitochondria, that it has oxidative phosphorylation ability, and that it contains the same nucleotides, and in the same proportions to each other, as are found in whole mitochondria.

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