ULTRAVIOLET INACTIVATION OF *EUGLENA* CHLOROPLASTS

I. EFFECT OF LIGHT INTENSITY OF CULTURE

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ABSTRACT Target numbers for ultraviolet (UV) inactivation of chloroplast replication in *Euglena gracilis* were about 45 when cells were grown in the dark and about 150 when grown in the light (700 or 1200 foot-candles [ft-cd]). Total cell DNA was about 25% greater in the light-grown cells.

INTRODUCTION

Replication of the plastid system in the protozoan *E. gracilis* is inactivated by low doses of UV light (Pringsheim, 1958). Epstein and Schiff, and a number of their coworkers, have studied this phenomenon in great detail (reviewed in Schiff and Epstein, 1968), and these studies were significant in leading to the demonstration of plastid DNA in *Euglena* (Brawerman and Eisenstadt, 1964; Edelman et al., 1964; Ray and Hanawalt, 1964). Inactivation analyses indicated that *Euglena* contained 30–40 UV-sensitive sites with respect to loss of plastid replication or "bleaching" (Hill et al., 1966 *b*). Since dark-grown *Euglena* contain about 30 proplastids (Epstein and Schiff, 1961), and UV inactivation was shown to be due to extranuclear damage (Gibor and Granick, 1962), it was logical to assume that each proplastid contained one UV-sensitive entity. *Euglena* grown in the light, with about 10 differentiated chloroplasts, also had inactivation numbers of 30–40. Thus, each chloroplast should contain three to four UV-sensitive entities.

E. gracilis is apparently polyploid under normal conditions of culture (Hill et al., 1966 *a*). Epstein and Allaway (1967) showed that DNA levels in *Euglena* could be halved if the cells were grown in continuous culture with phosphate or sulfate as a limiting nutrient. Using another approach, Parenti et al. (1971) found a five- to sixfold range in DNA levels in *Euglena* progressively starved for phosphate. Morimoto and James (1969) also found DNA levels in the related flagellate *Astasia longa* to be continuously variable over a sixfold range when sulfate was made a limiting nutrient. More importantly for the present thesis, Epstein and Allaway (1967) found that chloroplast numbers in *Euglena* containing half the normal complement of DNA were also halved; and UV target numbers for plastid replication dropped from about

40 to about 20. The implication is that total plastid DNA levels are proportional to total cell DNA levels.

This finding suggests a possible explanation for some discrepancies existing in the literature concerning inactivation (extrapolation, target) numbers for bleaching of *Euglena*. Petropulos (1964), for example, reported values of about 60, while Brody et al. (1965), using a theoretical, nonirradiation approach, estimated that there should be about 80. We agree with Hill et al. (1966 *b*) that the intrepretation of individual experiments is sometimes ambiguous, and a true target number of 40 (or 60) could easily be read as 60 (or 40) because this distance is not very great on a logarithmic scale and there is always some error involved in constructing such a curve. There remains, however, the possibility that these differences in extrapolation numbers are real and due to differences in culture conditions. In this paper light is shown to modify extrapolation numbers for UV inactivation of plastid replication. Light also modified total cell DNA levels, but the changes were not strictly proportional to the extrapolation numbers which were found for bleaching.

METHODS

E. gracilis Klebs (strain Z) Pringsheim was used throughout. The defined medium of Cramer and Myers (1952) containing 0.03 M ethanol as carbon source was used for liquid cultures (at pH 3.2, adjusted with H_2SO_4), while agar plates contained the same basal medium with 0.03 M acetate as carbon source (pH 6.8).

Liquid cultures were carried in Erlenmeyer flasks held in a lucite water table; circulating water held the temperature constant at 25°C. The table was lighted from underneath, at one end only, with 40-watt cool white fluorescent lamps. An intensity gradient was thus estabe lished along the table, ranging from 1200 to 150 ft-cd. Geometry of the flasks and lamps makthese intensity values approximate only, but they are correct to within 10%, as estimated with a Weston Photometer (Weston Instruments, Newark, N. J.). Dark-grown cultures were held in the same water table, with the flasks wrapped in several layers of aluminum foil.

Every effort was taken to make light intensity the only variable of culture. Thus, a single batch of medium was prepared and dispensed into the several flasks. The parent culture, to be used for inoculation, was grown in the dark, washed with water (to prevent carry-over of the old to the new medium), and equal numbers of cells added to each of the culture flasks. All harvests for irradiation and chemical analyses were made within a 24 hr period, when cultures had grown to 40–100 $\times$ 10^3 cells/ml, midlog phase growth under these conditions. None of these intensities of light inhibited growth. Any difference in UV sensitivity or chemical composition were therefore due to light intensity and not to history of culture. Growth was followed with the Coulter cell counter (Coulter Electronics, Industrial Div., Hialeah, Fla.).

Cultures to be irradiated were diluted approximately 10-fold with water, and these suspensions (10 ml) poured into glass Petri dishes (9 cm, total liquid depth about 1.5 mm). The total cell volume in this suspension was estimated to be no more than 0.004% of the whole, precluding mutual shielding. Approximately 0.25 ml was then transferred with a Pasteur pipette to a sterile plastic Petri dish (as the zero time control) and then the cell suspension placed under the UV light. Samples of about 0.25 ml were removed periodically and each placed in a plastic Petri dish. The cell suspensions were swirled regularly during irradiation. At the end of the irradiation period, all samples in plastic plates were flooded with 10-ml medium containing 0.8% agar. This latter medium was carried in metal-capped test tubes and kept liquid

by holding at 44°C. The agar solidified quickly at room temperature and the brief exposure to the elevated temperature apparently had no effect on greening ability of the cells. The irradiation was carried out in the dark and plates were stored in the dark for 1 wk to prevent photoreactivation (Lyman et al., 1959); they were then placed in 300 ft-cd light for 4 days to permit greening in competent colonies.

Irradiation was done with the middle 2.7 cm of a 15-watt germicidal lamp, major output at 2537 A, at a distance of 124.5 or 77 cm from the cell suspensions. These distances yielded dose rates of 7.76 and 20.3 ersg/mm² per min. The lower dose rate was used to increase precision in removing samples (every half-minute); the higher dose rate was used in some experiments in order to establish reciprocity.

Each plate was made to contain 2000–4000 cells, a number which insured that each colony would have been derived from a single cell. Colonies (1000-2000 per plate) were examined with the compound microscope, so that sectored colonies, which sometimes contained no more than 10% green cells, could be recognized and scored as green.

Cells grown in each of the light intensities were examined with the fluorescence microscope at the time of harvest, and chloroplast counts made as described elsewhere (Cook, 1972).

The balance of the cultures were harvested for chemical analyses. Cells were packed by centrifugation, washed three times by resuspension in water, and dispensed into 12-ml centrifuge tubes. The cells were then packed and the water decanted. Those to be used for nucleic acid determinations were brought to 0°C, mixed with cold 95% ethanol, and stored at −20°C. Those used for chlorophyll and protein analyses were immediately extracted three times with 95% ethanol at room temperature; these extracts were pooled, made to volume, and chlorophyll levels estimated by the difference in absorbance at 665 and 750 nm (Cook, 1972). The cells were further extracted with 95% ethanol at 60°C, solubilized with 1 N NaOH, and protein levels estimated with a biuret assay (Cook and Heinrich, 1968).

For nucleic acid analyses, cells were thawed at 0°C, and extracted twice more with 95% ethanol at 0°C and once at 60°C. Two methods were used to estimate DNA, UV absorption according to Schmidt and Thannhauser (1945) and the color reaction of Ceriotti (1952). For the former, lipid-extracted cells were extracted with ice-cold 5% perchloric acid (PCA) three times and then incubated overnight in 1 N NaOH at 37°C; the mixture was brought to 0°C and neutralized with an equal volume of 1 N HCl, and made to 5% in PCA; three further washes of the precipitate with cold 5% PCA were added to the first and the whole brought to volume to yield the hydrolyzed RNA. The precipitate was mixed with 5% PCA and held at 100°C for 20 min; the supernatant, with two washes, was made to volume to yield hydrolyzed DNA. Both RNA and DNA concentrations were estimated by the difference in absorbance at 260 and 315 nm.

Lipid-extracted cells to be assayed by the Ceriotti method were extracted three times at 0°C with 10% trichloroacetic acid (TCA) and then held at 100°C in 10% TCA for 20 min. The hydrolysate was made to volume and the procedure of Ceriotti (1952) followed thereafter. For both methods of assay, standard curves were constructed using salmon sperm DNA or yeast RNA (Schwarz Bio Research Inc., Orangeburg, N. Y.).

RESULTS

Two separate cell suspensions of dark-grown *Euglena* were irradiated, utilizing both the higher and the lower dose rates. The inactivation curves are shown in Fig. 1. A single regression line satisfies both sets of data, and extrapolation numbers in both experiments were the same, about 45, indicating that reciprocity in these dose rates existed for dark-grown cells.

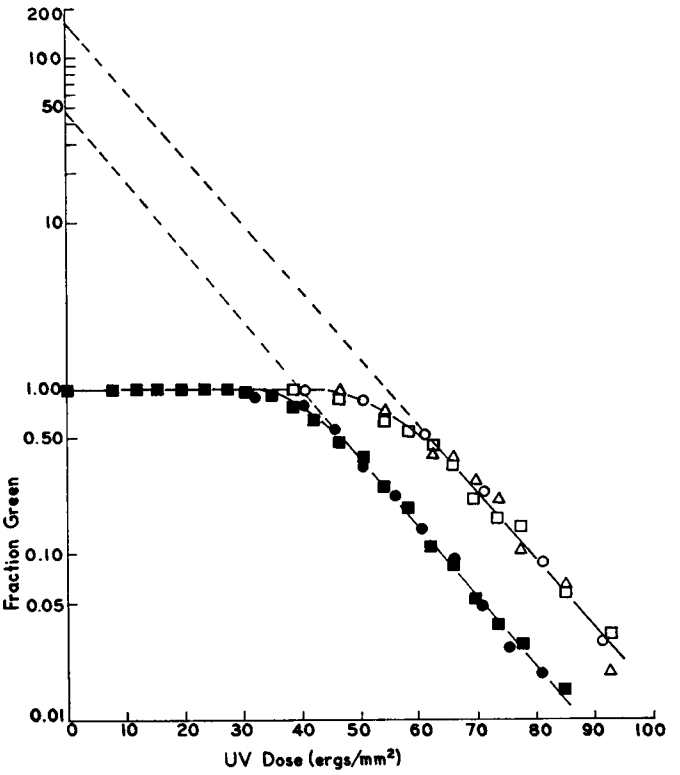

FIGURE 1 Inactivation of green colony-forming ability in *Euglena* grown in the dark or in two intensities of light. Filled symbols, cells grown in the dark; open circles and squares, grown in 1200 ft-cd; open triangles, grown in 700 ft-cd. Squares and triangles, irradiated at 7.76 ergs/mm² per min; circles, irradiated at 20.3 ergs/mm² per min. Ordinate shows the fraction of colonies able to green and (dashed lines) extrapolation numbers at the intercepts.

Two suspensions of cells grown at 1200 ft-cd were irradiated, one at each of the two dose rates. These inactivation curves are also shown in Fig. 1. Again, reciprocity was found to hold. The extrapolation number, however, was considerably greater than that found for dark-grown cells, being about 150.

One culture grown in 700 ft-cd light was irradiated at the lower dose rate. The inactivation curve, also shown in Fig. 1, did not differ from that found with cells grown in 1200 ft-cd light. Inspection of Fig. 1 shows that the extrapolation number for light-grown cells differs from that of the dark-grown cells largely because of a shift without appreciable rotation of the inactivation curves.

Light-grown cells contained the same number of chloroplasts, 8–10, regardless of the light intensity of culture (Table I). This is the normal complement of chloroplasts in this strain of *Euglena*. Chlorophyll levels per cell were inversely proportional to light intensity (Table I), showing that individual chloroplasts contained less chlorophyll when cells were grown in the higher light intensities. A more detailed analysis

TABLE I

BIOCHEMICAL CHARACTERISTICS OF *EUGLENA* AS A FUNCTION OF LIGHT INTENSITY OF CULTURE

	Light intensity (ft-cd)				
	Dark	150	300	700	1200
Chloroplasts/cell	—	8–10	8–10	8–10	8–10
Chlorophyll/cell (pg)*	—	9.04 (3)	9.17 (3)	5.41 (3)	5.94 (3)
Protein/cell (pg)*	266 (3)	420 (3)	385 (3)	385 (3)	382 (3)
RNA/cell (pg)*	27.3 (3)	45.2 (3)	42.7 (3)	44.4 (3)	41.7 (3)
DNA/cell (pg)*, ‡, §	3.48 ±0.14 (6)	4.62 ±0.26 (6)	4.37 ±0.31 (6)	3.96 ±0.21 (6)	4.14 ±0.20 (6)
DNA/cell (pg)*, ‡, ‖	3.42 ±0.54 (10)	4.78 ±0.41 (10)	4.85 ±0.26 (10)	4.40 ±0.31 (10)	4.31 ±0.21 (10)

* Number of replicate samples in parentheses.
‡ Mean ±SD.
§ By the method of Ceriotti (1952).
‖ By UV absorption.

of this relationship (in phototrophic cultures) is described elsewhere (Cook, 1972). Protein and RNA levels, however, were relatively invariant as a function of light intensity of culture, although values for light-grown cells were considerably greater than those found in dark-grown cells (Table I). It is firmly established that light stimulates synthesis of RNA and protein to levels well above those found in dark-grown *Euglena* (Brawerman et al., 1962). The degree of photostimulation is apparently independent of intensity, at least over the range reported in Table I and under the conditions used in this study. Under these conditions, photostimulation of RNA and protein synthesis was an all-or-none response.

Similar results, though less pronounced, were found in the DNA analyses. Total cell DNA, estimated by two different techniques, was about 3.5 pg per cell when *Euglena* was grown in the dark; but 4.4 pg per cell (range 4.0–4.8) when grown in the light.

DISCUSSION

Initiation of this study was suggested because of findings reported in two unrelated papers. Senger and Bishop (1966) showed that DNA levels in *Chlorella pyrenoidosa* increase with light intensity of culture. DNA levels were not strictly proportional to light intensity, but at least a sixfold range in DNA was observed and "saturation" was not found even at the highest intensity examined (21 klx, about 2000 ft-cd). Presumably most of this DNA is nuclear. Nevertheless, Epstein and Allaway (1967) found that extrapolation numbers for UV-inactivation of plastid replication in

Euglena were directly proportional to total cell DNA, when the latter was reduced by culture on limited nutrient supply.

Neither finding was realized in these studies with *Euglena*. DNA levels were increased slightly by visible light, perhaps by an average amount of 25% (Table I), but the intensity of light (over the range used in this study, 150–1200 ft-cd) was not proportional to the increase. Apparently light-stimulated DNA synthesis in *Euglena* is an all-or-none response with respect to light intensity.

Extrapolation numbers for UV-inactivation of plastid replication were not porportional to total DNA content. These numbers were at least three times greater for light-grown than for dark-grown cells. If it is assumed that the damaging action of UV is solely upon plastid DNA, then it follows that plastids of light-grown *Euglena* must contain at least three times more DNA than those of dark-grown cells. The strict proportionality between extrapolation numbers and total cell DNA found in the study reported by Epstein and Allaway (1967) apparently does not hold under all culture conditions.

Each of the chloroplasts in this study had average multiplicities of about 15 with respect to UV inactivation, compared with 3–4 found by the Brandeis group. In either case it is inferred that the chloroplasts are polyploid. Herrmann and Kowallik (1970) arrived at a similar conclusion with other material. The degree of ploidy is apparently subject to modification by environmental conditions. Gibbs et al. (1971) have presented evidence that light stimulates the synthesis of DNA in chloroplasts of *Ochromonas danica*. An earlier study from this laboratory suggested that this was also the case in *Euglena* chloroplasts (Cook, 1966). A severalfold change in plastid DNA levels would not contribute much toward change in total cell DNA; if plastid DNA in dark-grown cells is taken as 3% of the total (Edelman et al., 1964), a four-fold increase in this fraction would elevate total levels by about 10%.

Two other points of comparison between these data and those of the Brandeis group require emphasis. First, when our conditions of culture approximated theirs (i.e., low pH in the dark) extrapolation numbers were approximately the same. Our light-grown cells, however, yielded extrapolation numbers about four times greater than those found in the Brandeis laboratory. On the basis of results presented in this paper, the most logical explanation of this difference would relate to culture conditions. The Brandeis workers use a different medium and very low light intensities, about 100–150 ft-cd (Epstein and Schiff, 1961; Hill et al., 1966 *b*). This light intensity is adequate for chloroplast development (Epstein and Schiff, 1961), but well below minimal light levels required for optimal phototrophic growth (Cook, 1963) and may thus be well below optimal levels for full development of plastid DNA levels. In any event, we would agree with Brody (1968) that the extrapolation value of 40 reported by the Brandeis group may represent a lower limit for extrapolation numbers (during unlimited growth).

These interpretations rest on the assumption that plastid DNA is the primary

target of UV. It is recognized that other mechanisms may be involved, e.g. targets other than plastid DNA may be important in the UV interruption of plastid inheritance, or dark repair systems may be more active under some conditions of culture. These aspects of UV damage have not been considered in loss of plastid heritability in *Euglena*.

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