Action of Nalidixic Acid on Chloroplast Replication in *Euglena gracilis*[1]

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ABSTRACT

The role of light in nalidixic acid bleaching of *Euglena gracilis* var. *bacillaris* was investigated. The kinetics of loss of the chloroplast-associated DNA and the sensitivity of chloroplast replication to ultraviolet light was followed during treatment with nalidixic acid. By using the mutant P_4ZUL, and 3-(3,4-dichlorophenyl)-,1-dimethylurea, it was demonstrated that the requirement for light was a functioning photosynthetic electron transport system. Ultracentrifugal analysis showed a substantial decrease in chloroplast-associated DNA after 6 hours of treatment with nalidixic acid. Ultraviolet target analysis revealed that the number of chloroplast genomes per cell had been reduced. The possible role of light and implications of the reduction in chloroplast genomes for chloroplast replication are discussed.

Chloroplast replication in *Euglena gracilis* is specifically inhibited by Nal[2] (5, 6, 9, 13), a potent inhibitor of DNA synthesis (2, 7, 8). We have shown previously that Nal concentrations effective for the production of permanently bleached cells have no effect on cell division rate or viability although cell division is required for the Nal effect. Dark-grown cells, under resting conditions in the light, exhibit normal Chl synthesis in the presence of the drug (9). However, Neumann and Parthier (13) reported that dark-grown cells growing in Nal for 12 hr and then exposed to light show severe inhibition of Chl synthesis, RuDP carboxylase activity, and chloroplast-specific leucyl-tRNA synthetase as well as structural changes in the chloroplast. Previously, we (10) had found that the effect of Nal is most pronounced when the cells are growing in the light. We present data showing that the requirement for light is a functioning photosynthetic electron transport capacity. Further, we demonstrate the kinetics of loss of chloroplast DNA in the presence of the inhibitor and a concomitant increase in UV sensitivity of plastid replication.

MATERIALS AND METHODS

Growth Conditions. *Euglena gracilis* Klebs var. *bacillaris* Pringsheim, wild type, and mutant strains were maintained and grown for experiments on a defined organic medium at pH 3.6 (18). Incubation was carried out in gyrotary water bath shakers (New Brunswick) at 26 C $\pm$ 0.5 C. The cultures were illuminated with a bank of four 40-w cool white Westingthouse fluorescent bulbs supplemented by cool beam tungsten 150- and 75-w bulbs (General Electric) to give an intensity of 3.6 $\times$ 10^5 ergs/cm^2·sec.

Green colony-forming ability was assessed on plates using the above medium (18).

Preparation of Nalidixic Acid. A fresh stock solution of Nal[3] (5 mg/ml) was prepared for each experiment as follows: 100 mg of Nal powder were dissolved in 0.8 ml of 1 N NaOH and distilled H_2O added to 100 ml. The solution was sterilized by Millipore filtration. The final concentration of Nal for all experiments was 50 μg/ml of culture.

Preparation of DCMU. For the stock solution DCMU was dissolved in 95% ethanol. The final concentration for all experiments was 10^{-5} M.

DNA Isolation. A total of 3 to 4 $\times$ 10^8 log phase cells was harvested by centrifugation at 3,000 rpm in a Sorvall refrigerated centrifuge at 4 C. The resultant pellet was washed once with distilled H_2O followed by a second wash with saline-EDTA. Those cells not extracted immediately were fast frozen in Dry Ice and acetone then stored at -5 C.

DNA was isolated using a modification of the Marmur method (12). The pellet was suspended in a 5-ml cold saline-EDTA (0.15 M NaCl, 0.1 M Na_2EDTA, pH 8). SLS (25%, freshly prepared) was added to a final concentration of 0.2 mg/ml of suspension and lysis was determined by microscopic examination. If lysis was incomplete, the tube was warmed with the hands and/or a small additional amount of SLS added. The material was then incubated with 1 mg/ml pronase (stock solution 10 mg/ml, predigested) at 37 C. The lysate was placed in an ice bath, and 5 M sodium perchlorate was added to a final concentration of 1 M. One volume of chloroform-isoamyl alcohol (24:1, v/v) was added, and the suspension was placed in an ice bath for 30 min. During this time the emulsion was agitated frequently with a gentle reciprocal motion. Following this deproteinization, the material was centrifuged at 5,000g for 5 min (Sorvall HB-4 rotor). The supernatant fraction containing the nucleic acids was removed with the wide end of a 10-ml pipette into a chilled beaker. Two volumes of ice cold 95% ethanol were layered on the supernatant and the DNA was precipitated by gentle swirling. After winding the DNA onto a glass rod and repeated washings in cold ethanol, the fibers were dissolved in cold SSC (0.015 M NaCl, 0.0015 M Na_3 citrate, pH 7.4). Ribonuclease A (final concentration 50

[2] Abbreviations: Nal: nalidixic acid; P_4ZUL: a mutant designation (18); SLS: sodium lauryl sulfate; SSC: saline sodium citrate.

[3] Nalidixic acid was the generous gift of the Sterling-Winthrop Research Institute, Sterling Drug, Inc.

μg/ml) and ribonuclease T1 (final concentration 50 units/ml) were added, and the solution was then incubated for 30 min at 37 C. The beaker was then placed immediately into an ice bath. The DNA was reprecipitated by drop-wise addition of ice-cold isopropanol while gently swirling the beaker. The precipitate was washed repeatedly with isopropanol, air dried, and stored as fibers at -5 C.

Cesium Chloride Analysis. The DNA fibers were dissolved in SSC and an absorbance measured at 260 nm (20 A_{260} = 1 mg DNA/ml). A solution containing 0.6 g of CsCl, 20 μg of DNA, and 2 μg of marker DNA (*Micrococcus luteus,* Miles Laboratories, stock solution 40 μg/ml, stored at -20 C) in 0.47 ml of SSC was prepared for each determination. A similar solution minus DNA was used as a blank. The refractive index of each sample was adjusted to 1.398 (26 C) representing a density of 1.6850. The samples were placed in a double sector cell with a Kel-F centerpiece and installed in either an An-D or An-F rotor then centrifuged in a Spinco model E ultracentrifuge for 20 hr at 44,000 rpm at 20 C.

Ultraviolet Target Analysis. UV target analysis of chloroplast replication was done as described previously (11).

RESULTS

We have shown previously that cell division is required for the Nal effect and that green colony-forming ability in dark or light grown cells is lost rapidly in the light but is unaffected in the dark. Even after extensive growth in the dark in the presence of Nal, only a small fraction of the population ever shows inhibition of green colony-forming ability (9). The role of light in enhancing the Nal effect was investigated using P$_4$ZUL, a mutant of *Euglena,* which has a block in photosynthetic electron transport (14, 15) and DCMU, an inhibitor of photosynthetic electron transport. P$_4$ZUL, while capable of making chloroplasts in the light, is unable to photosynthesize. DCMU has no effect on chloroplast development (19). Figure 1 indicates the effect of Nal on the mutant and of DCMU plus Nal on the wild type. It can be seen that green colony formation is not inhibited by Nal when photoelectron transport is blocked in the plastid. Whether photosynthetic electron transport is blocked by an inhibitor or by a mutation, the result is a loss of sensitivity to Nal. It appears, therefore, that the light enhancement of the Nal effect is related to a functioning photosynthetic electron transport system.

In addition, the kinetics of the loss of ability to form green colonies in these experiments seem greater than can be accounted for solely by a dilution process. Complete loss of ability to form green colonies is seen with 4 generations, a faster rate than would be obtained by diluting out 10 chloroplasts (17). Since it has been shown that bacteria inhibited by Nal show degradation of DNA (3), we investigated the kinetics of chloroplast DNA loss in Nal-treated *Euglena.* Figure 2 shows that cells incubated for 6 hr in Nal show a considerable loss of chloroplast-associated DNA. In these experiments the mitochondrial DNA satellite peak is used as an internal standard. The loss of chloroplast-associated DNA occurs at a point in the bleaching curve where only about 20% of the population has bleached. This loss of chloroplast-associated DNA is too great to attribute to complete loss from only 20% of the population and no loss in the remainder.

UV target analysis of chloroplast replication was undertaken to determine whether the number of chloroplast genomes in the green survivors had been reduced. Ultraviolet analysis of chloroplast replication in *Euglena* has been used to estimate the number of chloroplast genomes per cell. In these analyses it is assumed that the UV target number (N) derived from experimental data or theoretical calculations represents the num-
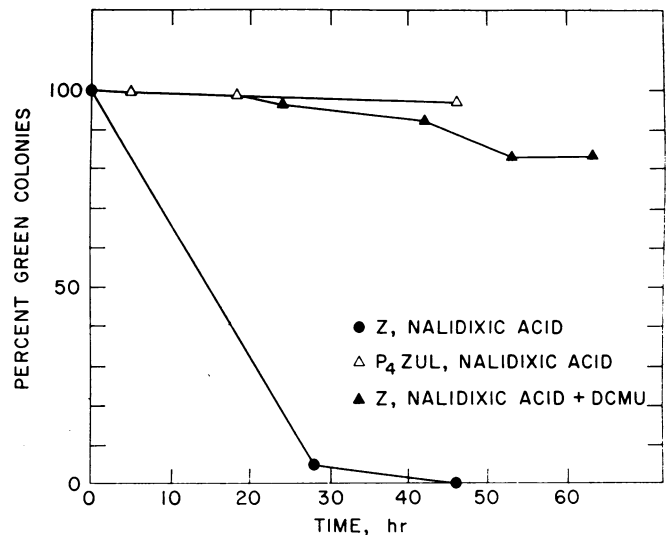
FIG. 1. Wild-type (Z) and mutant (P$_4$ZUL) cells were grown in the light in organic medium. Nal (50 μg/ml) and 10 μM DCMU were added as above.
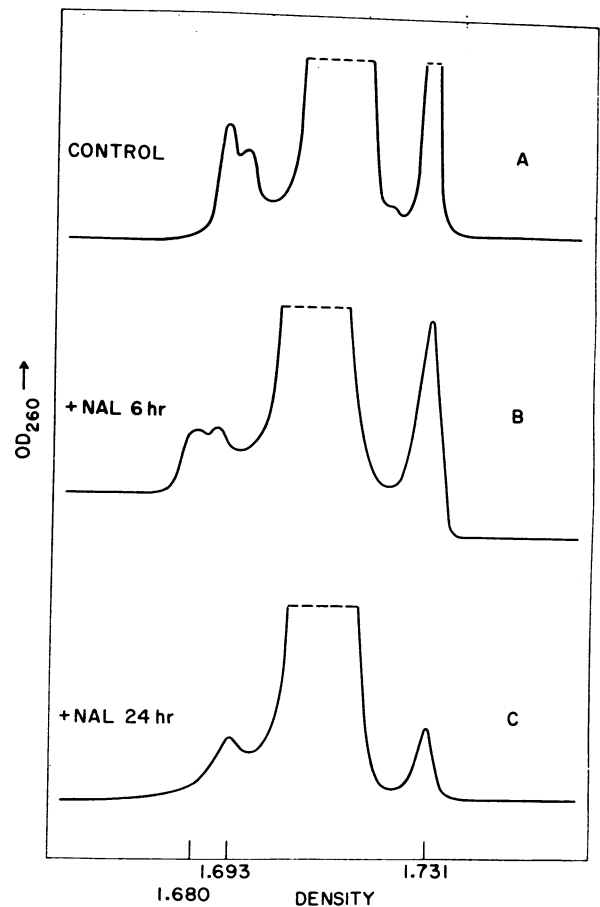

FIG. 2. Cesium chloride analysis of wild-type (Z) in the presence and absence of Nal. Regions of density indicate the following: 1.731, marker DNA (*M. luteus*); 1.709, *Euglena* nuclear DNA; 1.691, *Euglena* mitochondrial DNA; 1.686, *Euglena* chloroplast DNA.

ber of plastid genomes (11). It has been calculated that there may be 3 to 6 or more copies of the chloroplast genome (11, 20, 21); t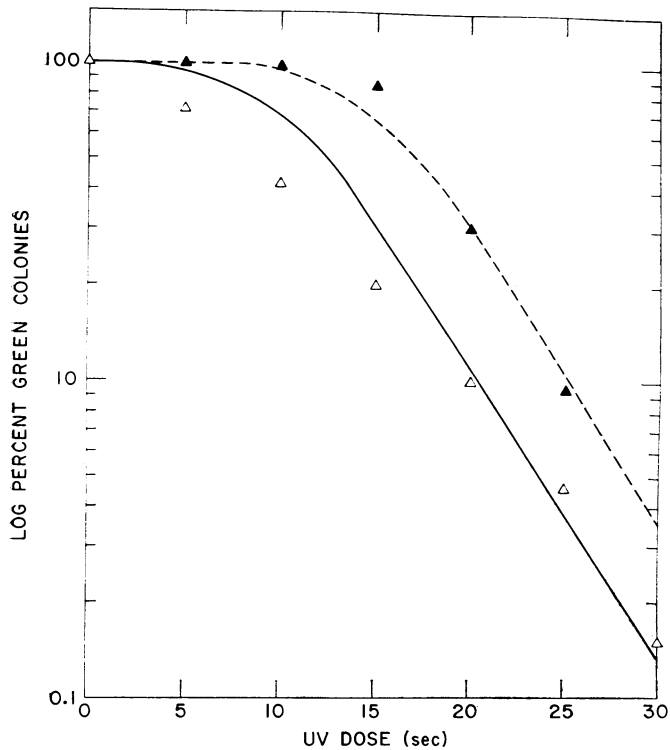herefore, there can be 30 to 60 plastid genomes per