SPECIFIC INHIBITION OF CHLOROPLAST REPLICATION IN *EUGLENA GRACILIS* BY NALIDIXIC ACID

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In *Euglena gracilis* the synthesis of the chloroplast from its precursor, the proplastid, is separated in time from the division or replication of the organelle which occurs just prior to cell division (10, 12). The proplastids are only found in dark-grown cells and rapidly form chloroplasts when such cells are exposed to light. The proplastids divide during dark growth. Ultraviolet irradiation inhibits proplastid division or replication but does not interfere with the proplastid's ability to synthesize chloroplasts (12). Chloroplast replication is also inhibited by ultraviolet irradiation (8). *Euglena*, then, is an excellent organism for the study of chloroplast synthesis and replication.

Nalidixic acid (1-ethyl 1–4 dihydro-7-methyl-4-oxo-1,8 naphthyridine-3-carboxylic acid) has been shown to be a specific inhibitor of DNA replication in certain bacteria, but to be bactericidal only if RNA or protein synthesis is allowed to proceed (4–6). We have found that this compound is a specific inhibitor of chloroplast replication in *Euglena gracilis* and that it has no effect on chloroplast synthesis or cell division. Proplastid replication is also inhibited but to a lesser degree. Inhibition of chloroplast synthesis markedly reduces the effect of nalidixic acid on chloroplast replication.

MATERIALS AND METHODS

Euglena gracilis, variety *bacillaris*, strain Z (Pringsheim), was grown on a rotary shaker with 500 footcandles fluorescent illumination (4–30-watt Sylvania F 30 T8-WW warm white bulbs) at 26°C in a defined heterotrophic acidic medium (7). For dark growth, cells were cultured in Erlenmeyer flasks covered with black plastic tape. Plating techniques were as described previously (8). Nalidixic acid was generously provided by Dr. Harry Pifer of Winthrop Laboratories, New York. A 25 ml stock solution was prepared by dissolving the compound in 1 ml N NaOH; the volume was adjusted with distilled water to a final concentration of 5 mg/ml, sterilized by filtration in a Millipore apparatus, and stored at −15 C. Cell counts were made with a Coulter Electronic Cell Counter. Chlorophyll was determined by Arnon's method (2). In this paper, the term dark-grown cells refers to *Euglena gracilis* cells which have been grown in complete darkness for many generations; these cells contain no chlorophyll or chloroplasts but have 30 or more proplastids and are capable of rapid synthesis of chloroplasts when exposed to light (10, 13). All manipulations of dark-grown cells were carried out with a safelight made from green Plexiglass (№2092, Rohm & Haas Co., Philadelphia, Pa.) over green fluorescent lamps.

RESULTS

Preliminary experiments indicated that a concentration of 50 μg/ml nalidixic acid in growing cultures of *Euglena* resulted in no loss of viability or change of growth rate, but large numbers of white colonies were seen when these cultures were plated. The growth of cells in light and darkness in the presence of nalidixic acid is shown in Fig. 1. There was no observed difference in rates of growth in untreated and treated cultures, and there was no loss of viability. Concentrations of nalidixic acid up to 200 μg/ml did not yield results substantially different from those obtained with 50 μg/ml. All experiments reported here were done with a concentration of 50 μg/ml.

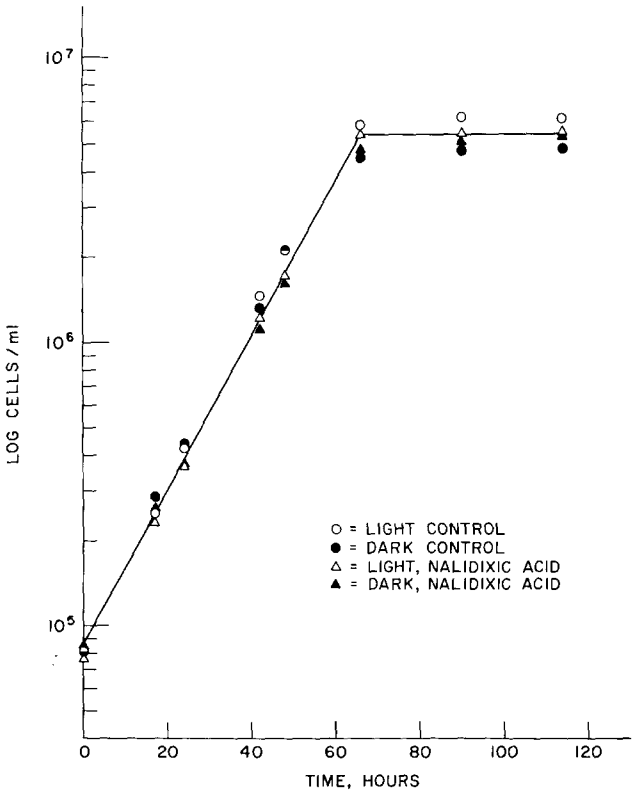

FIGURE 1 Effect of nalidixic acid on growth of *Euglena*. Cells were grown in 125-ml Erlenmeyer flasks on a rotary shaker with 500 footcandles fluorescent illumination. For dark growth, the flasks were covered with black plastic electrician's tape. Nalidixic acid concentration was 50 μg/ml. All manipulations of dark-grown cells were carried out in the light of a green safelight.

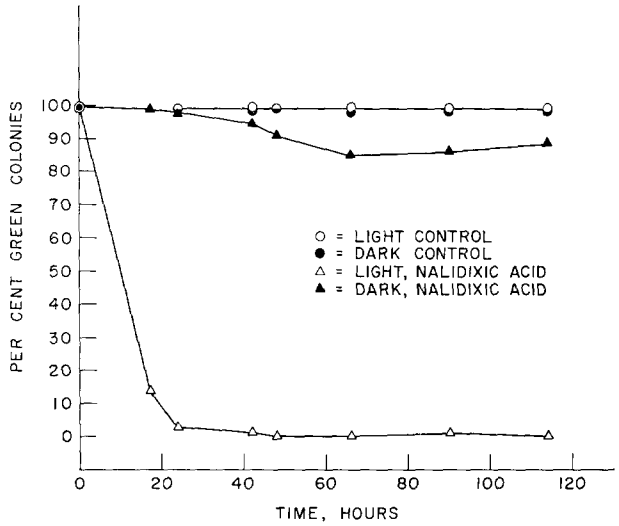

FIGURE 2 Effect of nalidixic acid (50 μg/ml) on green colony-forming ability. Cells were grown in light and darkness. At intervals, aliquots were plated in the light. No difference was seen between those plates placed in the light immediately upon removal from the growth flasks and those plates which were allowed to grow in the dark for 7 days and then placed in the light to allow colonies, capable of doing so, to form green colonies. Growth conditions as in Fig. 1.

The effect of nalidixic acid upon the ability of *Euglena* to form green colonies, when plated at intervals during growth in the presence of the drug, is shown in Fig. 2. Cells grown in the light with nalidixic acid rapidly lose the ability to form green colonies while dark-grown cells show only about a 15% loss. In *Euglena* the ability of dark-grown cells to form green colonies is indicative of proplastid replication in the dark (10).

The effect of nalidixic acid upon chloroplast synthesis was examined with dark-grown cells that had been washed and suspended for 48 hr in the

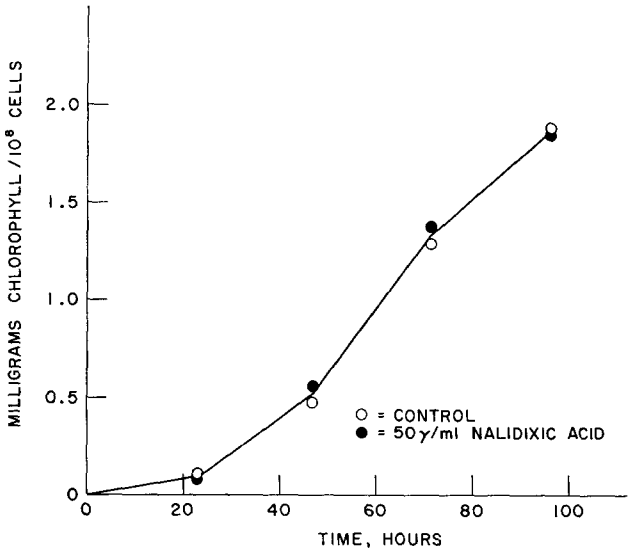

FIGURE 3 Effect of nalidixic acid (50 μg/ml) on chlorophyll synthesis in resting cells. Washed, nondividing, dark-grown cells were placed in the light on a rotary shaker.

dark in a medium that does not allow heterotrophic growth (13). The cells were then exposed to light and vigorous shaking. Cell division does not take place under these conditions, but rapid chloroplast synthesis ensues (13). Chloroplast synthesis, as measured by chlorophyll synthesis, is the same in treated and untreated cells (Fig. 3). The fully green cells were then washed and inoculated into autotrophic medium (7). That growth rate was the same for both treated and untreated cells indicates that the chloroplasts formed during exposure to nalidixic acid were fully capable of supporting photosynthetic growth. Cells that were removed during chloroplast synthesis, washed, and plated in the absence of the drug gave rise to only green colonies. Thus nalidixic acid had no effect on chloroplast synthesis in resting cells and was only effective in inhibiting green colony formation if cells were grown in the presence of the drug.

Euglena gracilis has 30 or more proplastids, when grown in the dark, and forms 10–12 chloroplasts upon exposure to light (10). The apparent low sensitivity of dark-grown cells to nalidixic acid (Fig. 2) could result from a "dilution" phenomenon, whereby more cell generations would be required to "dilute out" the proplastids than the less numerous chloroplasts. Cells were grown for more than 12 generations in the dark in the presence of the drug. Even with prolonged dark growth, however, dark-grown cells never lose more than 40% of their ability to form green colonies (Fig. 4). Concentrations of nalidixic acid up to

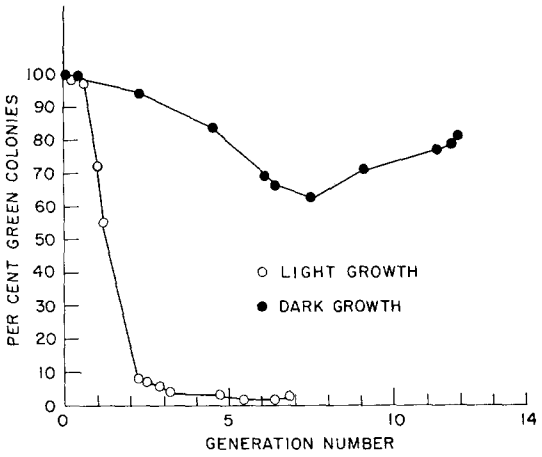

FIGURE 4 Per cent green-colony formation of cells grown for a prolonged period in the dark with nalidixic acid. Dark-grown cells were grown for six cell-generations in the presence of nalidixic acid (50 μg/ml) and then were transferred for six more generations to fresh medium also containing the drug. At intervals, aliquots were removed for plating to determine green colony-forming ability. A parallel culture was grown simultaneously in the light for seven generations. Growth conditions as in Fig. 1.

200 μg/ml did not increase the proportion of permanently white colonies in dark-grown cultures. They also exhibit a "recovery" phenomenon, and at the end of the experiment only 20% of the cells were incapable of forming green colonies.

Recovery of green colony-forming ability was never seen in light grown cultures, even after prolonged growth.

Protein and ribonucleic acid syntheses are prerequisites for the bactericidal action of nalidixic acid (5). The necessity for chloroplast protein synthesis in *Euglena* as a prerequisite to nalidixic acid effect on chloroplast replication was investigated. Chloroplast protein and ribonucleic acid syntheses are stopped by placing cells in the dark or by using inhibitors (13). Cells growing in the light in the presence of nalidixic acid were placed in the dark at various intervals and allowed to continue growth in the presence of the drug. In Fig. 5 it can be seen that removing cells from the light inhibits the effect of nalidixic acid.

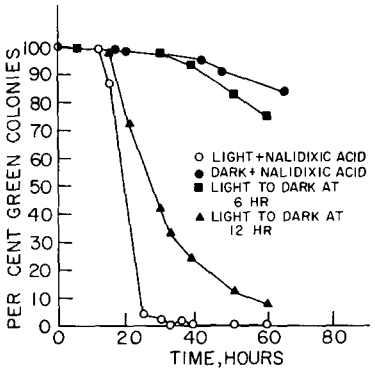

FIGURE 5 Inhibition of the effect of nalidixic acid by dark growth. At zero time, cells were inoculated into medium containing nalidixic acid (50 μg/ml) and were allowed to grow in the light. A control was grown in the dark. At 6 and 12 hr, aliquots were removed from the light culture and allowed to continue growth in nalidixic acid in the dark. All cultures were plated at intervals. Growth conditions as in Fig. 1.

An inhibitor of chloroplast protein synthesis was also utilized to determine whether chloroplast protein synthesis is necessary for the action of nalidixic acid. Ethionine acts as a specific inhibitor of chloroplast protein synthesis and chlorophyll synthesis in *Euglena* without affecting chloroplast or proplastid replication, cell division, or viability (1). Cells grown in the presence of ethionine are still able to give rise to the same proportion of green colonies as do untreated controls, but the former almost lost the capacity for chlorophyll synthesis (Fig. 6). When ethionine is added to cells growing in nalidixic acid, a marked inhibition of the effect of nalidixic acid is observed (Fig. 6). Experiments with dark-grown cells indicate that ethionine also inhibits the nalidixic acid effect on green colony-forming abilities of dark-grown cells.

DISCUSSION

The mode of action of nalidixic acid on *Euglena gracilis* chloroplasts is probably analogous to its action on bacteria. DNA is present in the chloroplasts (and the proplastids) of *Euglena* and has been implicated in the genetic continuity of the chloroplast (3, 9, 10, 11, 12). It is likely that nalidixic acid specifically inhibits the replication of this DNA.

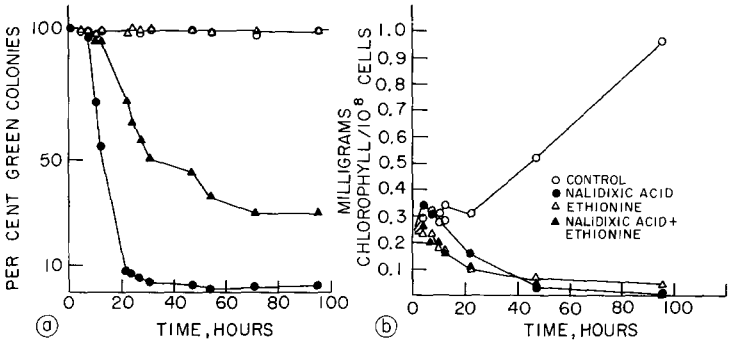

FIGURE 6 Effect of ethionine on nalidixic acid inhibition of green colony formation. (*a*) Effect of nalidixic acid on green colony-forming ability in the presence of ethionine. At zero time, light grown cells were inoculated into medium containing nalidixic acid (50 μg/ml) or ethionine (10 μg/ml) or nalidixic acid + ethionine. All cultures were grown in the light under the conditions of Fig. 1. (*b*) Chlorophyll synthesis under the conditions in *a*. The increase of chlorophyll per cell seen in the control is the usual increase seen in the later stages of growth in cultures of *Euglena* and is probably related to the effects of starvation on chloroplast synthesis (13).

The different responses of dark- and light-grown cells to nalidixic acid is difficult to interpret. Pro plastids and chloroplasts may differ in their permeability to the drug, or chloroplast DNA may be more susceptible to nalidixic acid than proplastid DNA, but a fourfold increase in nalidixic acid concentration did not yield more white colonies in dark-grown culutres. In *Bacillus subtilis* it was noted that exposure to nalidixic acid resulted in inhibition of DNA replication and degradation of the DNA (4). In other organisms treated with ultraviolet light and other agents which stop DNA replication it has been demonstrated that there is a degradation of the DNA by endogenous nucleases (14, 15). Perhaps nucleases specific for chloroplast DNA are synthesized during chloroplast formation and degrade the nalidixic acid-inhibited DNA. Alternatively, chloroplast DNA treated with nalidixic acid may be more susceptible to preexisting nucleases than proplastid DNA similarly treated. There is no evidence, however, to indicate that chloroplast DNA is different from proplastid DNA.

If the drug were tightly bound to a portion of the 30 proplastids in a dark-grown cell and thus inhibited their replication, then prolonged growth in the dark could result in the loss of the nalidixic acid–containing proplastids; this allows the remainder to give rise to normally replicating chloroplasts. The "recovery" phenomena would then be explained by the increase, during cell division, of cells in the population containing drug-free proplastids. It is difficult to see why some proplastids would bind nalidixic acid and others would not; however, it is not known whether all the proplastids within a cell of *Euglena* are identical.

The specificity of the drug for the chloroplast and the apparent lack of any effect on rate of cell division on viability may be a reflection of the differences between chloroplast DNA and nuclear DNA in *Euglena* (9), or a difference in permeability of the organelles to the drug.

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