DNA DEPENDENCE OF PLASTID DIFFERENTIATION INHIBITION BY ACTINOMYCIN D

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Euglena gracilis offers a particularly suitable system for investigating some of the fundamental processes involved in the provision, transmission, and translation of information required for the building of specific cytoplasmic structures. Cells grown in the dark do not have a defined chloroplast structure; they are etiolated cells (1). Light induces profound changes in the morphology and physiology of the cells leading to the formation of chloroplast structures. There is a significant increase of total pro-

tein and RNA with the appearance of specific plastid ribosomes and specific chloroplast proteins (2–4). Treatment of *Euglena* cells with streptomycin, heat, or UV irradiation causes "bleaching," *i.e.*, permanent loss of the capacity to develop chloroplasts. There is some evidence that nucleoprotein particles probably associated with a proplastid bear the necessary genomic information to affect differentiation into chloroplasts independently of the nucleus (5).

The aim of the present work is to find out whether the information required for the greening process is DNA dependent. For this purpose, we have used actinomycin D, a highly specific inhibitor of RNA-DNA polymerase activity (6). Actinomycin effect is exerted on the template by complexing specifically and firmly with the primer DNA. It has been shown in tissue cultures (7), in bacteria (8), and in plants (9), that actinomycin D inhibits almost completely the DNA-dependent synthesis of RNA.

MATERIAL AND METHODS

Euglena gracilis strain Z was used. Cells grown in the light and containing chloroplasts will be referred to as green cells. Those grown in the dark, devoid of chloroplasts but still capable of forming them, will be called etiolated, and those with a permanent loss of the capacity to produce chloroplasts will be designated bleached.

Media

Different media were used: (*a*) Complex medium (CM) containing 0.5 per cent proteose-peptone, 0.2 per cent yeast extract, and 0.1 per cent glucose (10); (*b*) Semisynthetic medium (SMM) (11); and (*c*) synthetic medium (SM) containing glutamic acid, malic acid, vitamin B_{12}, thiamine, and mineral mixture (12).

All cells were grown in these media at 24°C; etiolated cells were kept in the dark and green cells in the light. For studying the greening process, the cells were incubated in maintenance medium (MM), consisting of 1.0 per cent acetate, 0.01 M buffer phosphate pH 6.5 and 0.01 M $MgCl_2$, which completely prevents cell multiplication. Cells in this medium are kept in a water bath with permanent illumination and continuous shaking to maintain them in suspension.

Solid medium consists of 1.5 per cent agar, 1.0 per cent proteose-peptone, 0.4 per cent sodium acetate, and 0.4 per cent yeast extract. The plating procedure was performed as indicated elsewhere (13). Colonies were counted 7 to 8 days after plating.

Determination of Chloroplast Formation

Chloroplast production was determined by the chlorophyl concentration per 10^7 cells. There is a perfect correlation between amount of chlorophyl and number of chloroplasts (14, 15). Samples were taken at different times, and aliquots were used for cell counts in a hemocytometer after fixation with 5 per cent formaldehyde. Chlorophyl determination was performed in aliquots washed with distilled water; pigments were extracted with 80 per cent acetone and measured colorimetrically at 663 mμ and 645 mμ (16).

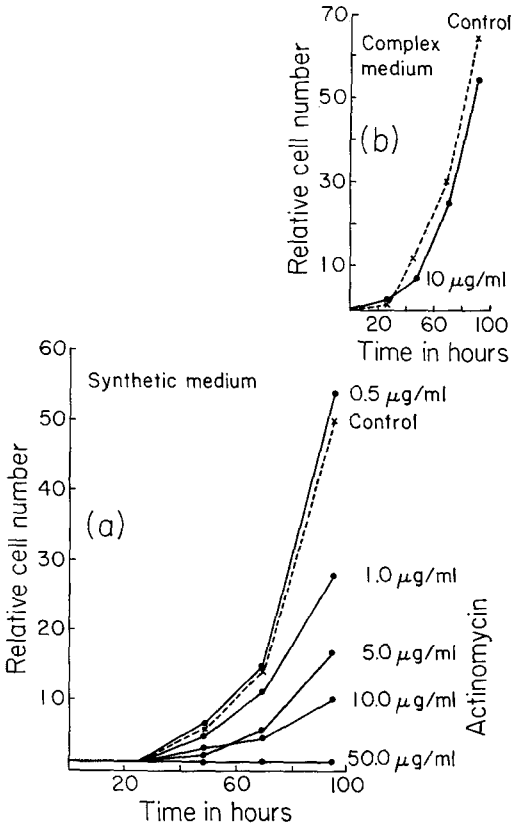

FIGURE 1 Effect on cell division at different actinomycin concentrations in SM (*a*) and CM (*b*). Cells grown in the light at constant temperature 24°C for 96 hours.

Determination of RNA Synthesis

Ribonucleic acid synthesis was measured by 2-^{14}C-uracil incorporation into total RNA. The radioisotope was suspended in distilled water, sterilized by passing the solution through a Millipore filter, and

inoculated into the *Euglena* cultures. Samples of cells were harvested and washed with distilled water. The pigment was extracted with 80 per cent acetone for chlorophyl determination and the residue was extracted with alcohol at room temperature and then treated twice at 0°C with 5 per cent trichloroacetic acid for 1 hour; the precipitate was washed with ethanol at room temperature, defatted with alcohol-ether (3:1) in a boiling water bath for 1 to 2 minutes, and dried with ether. This procedure insures complete extraction of any acid and alcohol-soluble fraction. The fine dry powder obtained was hydrolyzed with 0.3 N KOH at 37°C for 18 hours. The alkali hydrolysate was centrifuged to eliminate any starch particles, and the supernatant was brought to pH 4–5 with concentrated acetic acid. This causes precipitation of DNA and protein. Aliquots of the acid solution were spread and dried on planchets for radioactivity determinations with an end-window counter. RNA concentration was determined by UV spectrophotometry at 260 to 310 mμ.

Determination of Protein Synthesis

The kinetics of protein synthesis during the greening process were measured by DL-^{14}C-leucine incorporation into total cell protein using the same conditions of medium and sterilization as indicated previously. Acid and alcohol extraction was performed as indicated for RNA determinations. The fine dry powder residue was treated with 5 per cent trichloroacetic acid at 90°C for 20 minutes for nucleic acid extraction. The trichloroacetic acid precipitate was washed successively with alcohol and alcohol-ether (3:1) and dried with ether. The protein residue was

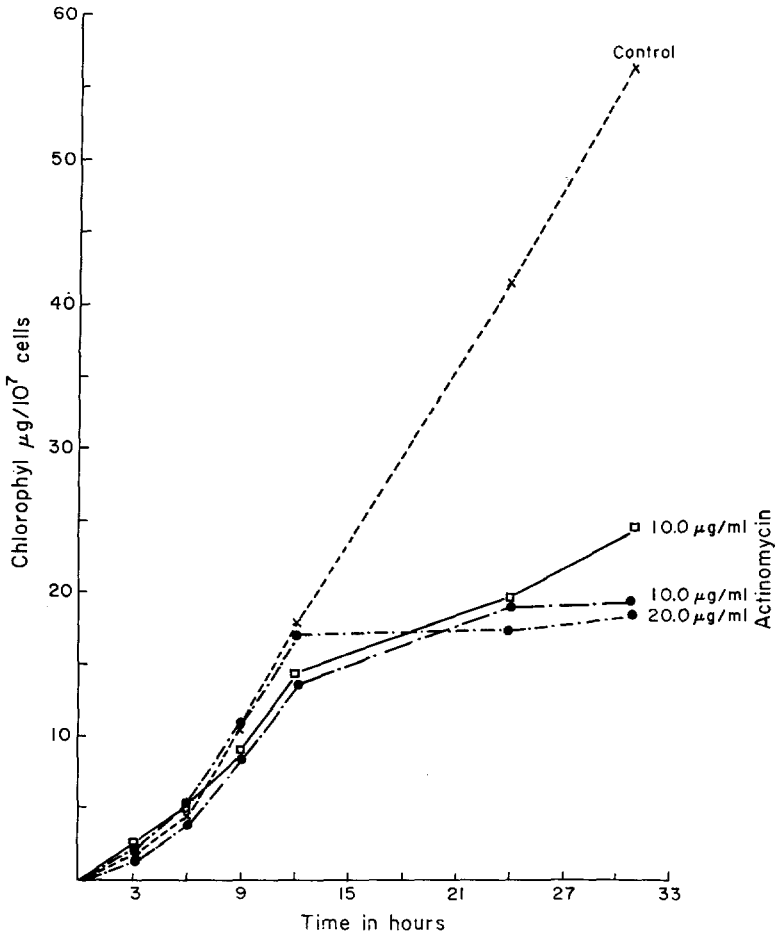

FIGURE 2 Actinomycin effect on the first stages of the greening process. Dark-grown cells in SMM, collected at stationary phase of growth, washed, and incubated in MM with actinomycin for 14 to 16 hours in the dark. Afterwards transferred to the light in actinomycin-free MM (-.-.-) and with the drug in MM (□—□).

dissolved in 1 N NaOH; aliquots were spread on planchets and counted in an end-window counter. Protein determination was made by a modified biuret method (17).

RESULTS

Actinomycin Action on Cell Proliferation

The effects of progressively increasing concentrations of actinomycin on growing cultures is shown in Fig. 1. The experiment was started with a cell density of approximately 5.0 to 8.0 × 10^4 cells/ml. It is seen that relatively high doses are necessary to inhibit cell division (8), and doses of 50 μg/ml produce complete growth inhibition and non-viable cells. 10 μg/ml are sufficient to inhibit cell multiplication by 80 per cent in SM (while leaving the cells fully viable). The same dose was ineffective in CM at a cell density of 2.0 × 10^4 per ml, which is half that used in SM. This could be due to inactivation of the drug by some component of the CM. Therefore, in all further experiments the cells were grown in semisynthetic or synthetic media.

Actinomycin Action on Greening Process and "Bleaching" Effect

The greening process implies the differentiation of proplastids into chloroplasts. The effect of actinomycin on this process was studied under conditions where there is no cell division. The MM supports active chloroplast formation without cell multiplication. Colorless cells grown in SMM in the dark were collected in the stationary phase, and washed twice with distilled water under sterile conditions. They were then incubated in MM with 10 μg of actinomycin per ml in the dark for 14 or 16 hours. Afterwards, the flasks were transferred to an illuminated water bath at 24°C with continuous shaking. The effect of the drug on chlorophyl synthesis during the first stages of the greening process in the light is shown in Fig. 2. At the beginning of light induction, both control and actinomycin-treated cells have exponential curves of chlorophyl synthesis. After about 12 hours, chlorophyl synthesis decreases and stops in the actinomycin-treated cells. The same effect is obtained whether the cells are cultured in the presence of actinomycin or whether they are previously treated with it for 14 or 16 hours, washed, and cultivated in actinomycin-free media. In either case, and even at higher drug concentration (20 μg/ml), the drug does not block chloroplast formation immediately.

The development of chloroplast is accompanied by a large increase in the protein content of the cells and by the appearance of a specific plastid ribosome (2–4). Although there is a considerable turnover of RNA and proteins in cells incubated in MM in the dark, light exerts a strong stimulation of radioisotope incorporation into these two fractions (18). In order to see whether actinomycin inhibits these chloroplast components, its action was studied on the synthesis of RNA and protein. Fig. 3 shows that actinomycin inhibits 2-^{14}C-uracil incorporation into total RNA fraction. With 10 μg/ml there is incomplete inhibition of RNA synthesis (also with 20 μg/ml). Fig. 4 shows that actinomycin also inhibits protein synthesis under the same experimental conditions. It can be con-

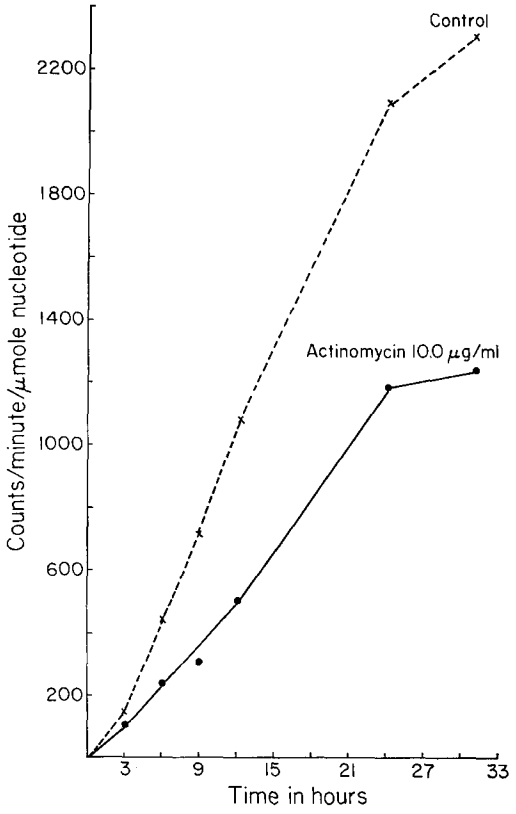

FIGURE 3 See Fig. 2 for experimental conditions. When cells were transferred to the light in actinomycin-free medium, the medium was supplemented with 1.0 μc/ml of 2-^{14}C-uracil, specific activity 10.2 mc/mM.

cluded, therefore, that actinomycin interferes with RNA, protein, and chlorophyl synthesis during the greening process, producing a blockage of proplastid differentiation into chloroplasts.

Euglena cells, after 96 hours of growing in the light in SM and in the presence of actinomycin, were collected, washed, and plated in agar to see whether bleached colonies were produced. With concentrations up to 10.0 μg per ml there is no difference, between control and actinomycin-treated cells, in the production of bleached colonies or in the viability of the cells. Therefore, the drug

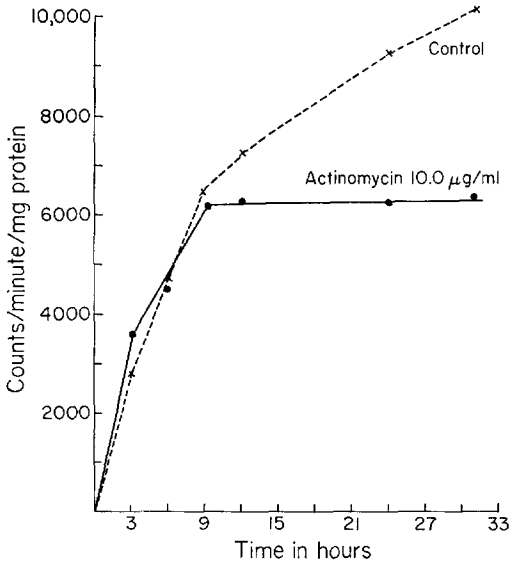

FIGURE 4 See Fig. 2 for experimental conditions. When cells were transferred to the light in actinomycin-free medium, 0.5 μc/ml of DL-1-^{14}C-leucine (specific activity 7.2 mc/mM) was added.

does not interfere selectively with the mechanism of self-reproduction of the proplastid.

DISCUSSION

The results presented in this report suggest that RNA, pigment, and protein syntheses connected with the greening process are inhibited by actinomycin. Chloramphenicol, under the same experimental conditions, prevents only the synthesis of protein and pigments, leaving the synthesis of RNA unaffected (19). Since actinomycin has a specific action on the DNA templates, our results indicate that the greening process is under DNA control. The information for the construction of a chloroplast structure thus appears to be stored in DNA molecules. With 10 μg/ml, the arrest of RNA production appears to be incomplete and stops after protein synthesis is inhibited. This result contradicts what would be expected if chloroplast development in *Euglena* involves DNA-dependent RNA synthesis followed by protein synthesis. However, any speculation about our data must take into consideration the following facts:

(*a*) It is not possible to establish any sequence between RNA and protein syntheses without evidence about the kinetics of uracil and leucine incorporations.

(*b*) The whole cell RNA and protein turnover probably masked the specific synthesis of the polymers.

(*c*) Since incorporation of ^{14}C-uracil into actinomycin-treated cells is markedly increased in di- and triphosphated nucleosides (20), terminal addition (21) or non-specific binding (22) cannot be ruled out.

Actinomycin has no effect during the first 12 hours of exposure in the light. This lag period of no-inhibition is apparently not due to permeability factors because the cells were in contact with the drug in the dark for a long time before greening started. It was impossible to produce immediate blockage either by leaving the drug in contact with cells during the whole period of light induction or by increasing the actinomycin concentration and the time of exposure in the dark. The formation of the chloroplast structure can proceed for prolonged periods in the absence of any genetic information which might provide a supply of "messengers." Therefore, the greening process could be included in the well known long-lived RNA template systems such as enucleate amoebae, enucleate *Acetabularia*, and mammalian reticulocytes.

The cytoplasmic localization of the proplastids (6) and the fact that specific DNA molecules were found in chloroplasts of broad bean (23) and *Euglena* (24, 25), and DNA-containing areas in chloroplasts of *Chlamydomonas* (26), raise the possibility that the DNA template for chloroplast formation is non-chromosomal. Brawerman and Chargaff (13) established that a cytoplasmic system which can replicate itself independently of cell proliferation bears some information for the greening process. In the light of our results, it may be proposed tentatively that this autonomous system has its own specific DNA templates. These complex units are independent of the rest of the cell in the

sense that they are self-informing both in their multiplication and in effecting the differentiation of chloroplasts.

SUMMARY

Actinomycin D inhibits chlorophyl, protein, and RNA syntheses associated with the greening process in *Euglena gracilis*. This effect affords evidence of a DNA-dependence in plastid differentiation.

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Note Added in Proof

Since this report was submitted there have been several papers published pertinent to the occurrence of chloroplast DNA and actinomycin D effect on greening.

KIRK, J. T. O., *Biochim. et Biophysica Acta*, 1963, **76**, 417.

CHUN, E. H. L., VAUGNAN, M. H., and RICH, A., *J. Molec. Biol.*, 1963, **7**, 130.

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The last one deals specifically with actinomycin D on *Euglena* chloroplast formation.