Studies of Chloroplast Development in Euglena
XI. Radioautographic Localization of Chloroplast DNA[1]

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Considerable evidence exists which is consistent with the picture that the chloroplast system of *Euglena* is under autonomous genetic control. This includes studies of ultraviolet inactivation and photoreactivation of chloroplast inheritance (7, 10, 13, 14) and the demonstration of a unique species of chloroplast-associated DNA (3, 4, 5, 9, 11). Evidence has been presented for distinct chloroplast-associated RNA, in addition to the usual nuclear DNA and cytoplasmic RNA (2, 6).

In this communication we provide additional evidence for the localization of DNA in the chloroplasts.

Materials and Methods

Euglena gracilis var. *bacillaris* was grown on Hutner's pH 3.5 medium (8) as described previously (10). For the described experiments, light-grown cells were inoculated into 125 ml of medium contained in 250-ml Erlenmayer flasks containing 1 ml of purine solution (see below) which had been adjusted to pH 3.5 prior to autoclaving. Flasks were incubated under approximately 400 ft-c of fluorescent light at 26°.

Cells to be incubated with radioactivity were first adapted to 1-week's growth on equivalent concentrations of nonradioactive purine before being inoculated into fresh medium containing the radioactive purine. In the case of guanine, the purine solution used contained 0.274 mg/ml 1N HCl. For radioactive experiments an identical quantity of guanine was dissolved which was also equivalent to 1 mc of tritium radioactivity. For experiments with adenine, 1 mc of adenine/ml water was added to the medium in addition to radioactive guanine. About 5×10^5 cells were inoculated into each flask and samples were removed at various times from 24 to 240 hours.

Guanine-H^3 (333 mc/mM) and adenine-H^3 (243 mc/mM) were purchased from Nuclear Chicago; their unlabeled counterparts were purchased from Nutritional Biochemicals.

Nucleic acids were isolated by Marmur and Doty's method with slight modifications (4). Samples were taken from all fractions in the isolation and radioactivity measured in dioxane scintillation fluid (770 ml dioxane, 770 ml toluene, 460 ml absolute ethanol, 1 g PPO, 0.1 g POPOP, 16 g napthalene) in a Packard Tri-Carb Model 314-DC scintillation spectrometer.

The DNA fraction was further treated with crystalline deoxyribonuclease (Worthington Biochemicals, 500 μg/ml containing 0.003 M $MgSO_4$) at 38° for 90 minutes. It was then dialyzed against 2 liters of standard saline citrate (0.15 M NaCl containing 0.015 M sodium citrate, pH 7.1).

Preparative cesium chloride gradients were carried out as described previously (4). Samples collected dropwise from a puncture in the bottom of the centrifuge tube were diluted with 1 ml of standard saline citrate solution for measurement of absorption at 260 mμ. These samples, after receiving 100 μg of yeast DNA as carrier, were made to 5 % trichloroacetic acid, held at 4° for precipitation and were filtered on 0.45 μ 25 mm Millipore filters. These were then dried and were added to toluene scintillation fluid (400 ml toluene, 40 mg POPOP, 2 g PPO) in vials for counting.

For radioautography, a drop of cell culture was placed on an acid-cleaned slide coated with Haupt's adhesive. An alcohol-cleaned coverslip bearing a drop of 50 % methanol was inverted over the drop of cell culture. This sandwich was then frozen on a block of dry ice and the coverslip peeled off. The slides were then placed successively in 3:1 ethanol-glacial acetic acid, 100 % ethanol and 95 % ethanol to remove the acid soluble materials and were air dried and reserved at 4° for enzyme treatment.

For treatment with deoxyribonuclease, slides were placed in a solution containing 0.1 mg/ml crystalline Worthington deoxyribonuclease in 0.003 M $MgSO_4$ adjusted to pH 6.6 with NaOH for 4 hours at 38°. Ribonuclease treatment employed 0.2 mg/ml Worthington crystalline ribonuclease in water at pH 6.6 for 4 hours at 38°. In all cases, control slides were treated identically, with the omission of enzyme. In some cases, slides were treated with both enzymes at these concentrations simultaneously; in others the 2 solutions were used sequentially.

After washing and air drying, the slides were coated with Ilford L-4 emulsion at a dilution of 2:1 emulsion; water at 45°. They were then exposed at 4° for 7 to 8 weeks in light-tight boxes and were then developed with Kodak D19 for 5 minutes followed by fixation. The pattern of label was observed with a Zeiss phase contrast microscope. To increase contrast for photography some preparations were stained through the emulsion with iron hemato-

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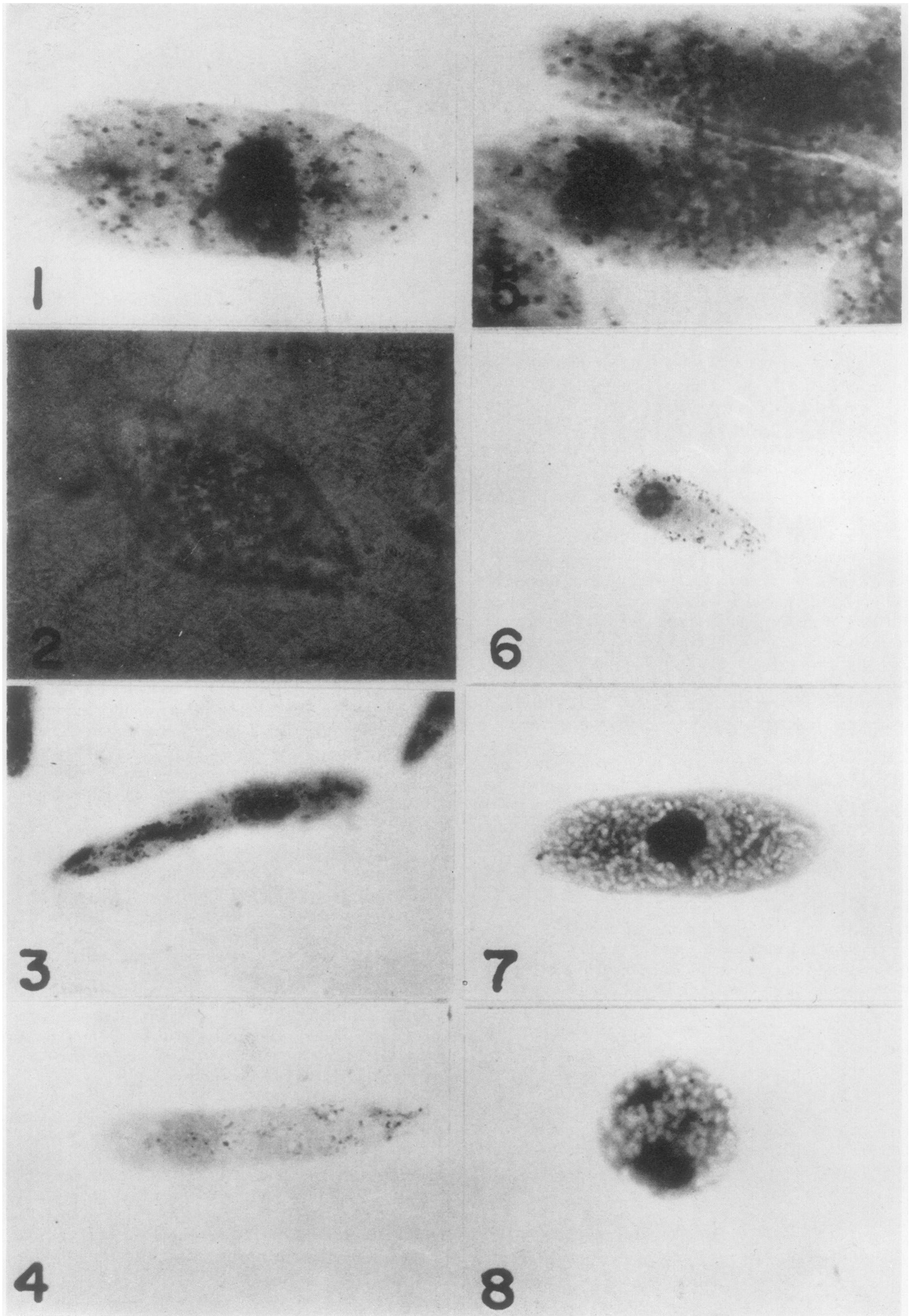
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xylin. For this, the slides were mordanted for 2 hours at room temperature in 30 g/liter ferric ammonium sulfate containing 10 ml glacial acetic acid and 1.2 ml sulfuric acid. After washing, they were stained with 0.5 % aqueous hematoxylin for 2 hours or longer until chloroplasts and nuclei showed overstaining. The slides were then destained with 0.5 concentration of mordant solution for about 30 seconds or until good differentiation of plastids and nuclei were observed. Slides were then washed, air dried and observed for grains over the stained areas.

Results and Discussion

Chemical Localization of Label in Cell Fractions. Since Sagan (12) and others have shown that thymidine, and pyrimidines in general, are not effective precursors of DNA in *Euglena*, explorations with a purine, guanine, were undertaken based on the report of Bolton et al. (1). As may be seen from table I, the label from tritiated guanine appears in the nucleic acids of cells which were exposed to guanine-H^3. On lysis of the cells for DNA extraction after guanine-H^3 incorporation, virtually no counts were found in the organic layer and protein precipitate at the first Sevag deproteinization. About 5 % of the label in the medium appears in the total nucleic acids with about 20 % of this in DNA (specific activity = 340 cpm/μg DNA). In other experiments, mixtures of adenine and guanine were used in an attempt to increase the amount of label incorporated, but added adenine-H^3 did not significantly increase the total amount of nucleic acid labeling. Other unsuccessful attempts to increase the total amount of incorporation included increased molar concentrations of guanine-H^3, prolonged incubation times, and preincubation with label under nondividing conditions. These did not significantly enhance the specific activity of the nucleic acids.

Table I. *Uptake of Guanine-H^3 into Nucleic Acid Fraction*

Total counts in incubation medium	1.8×10^7
DNA + RNA*	8.7×10^5
DNA**	1.3×10^5
After DNAase***	297

* After first Sevag deproteinization.
** After 2 additional Sevag deproteinizations followed by ribonuclease treatment.
*** After 1 additional Sevag deproteinization, deoxyribonuclease treatment and dialysis.

To ensure that the label is indeed in the forms of DNA ordinarily encountered in *Euglena* (4, 5) the DNA fraction obtained above was subjected to density gradient centrifugation in cesium chloride (4). The major peak centering at drop 15 in figure 9 is at the usual position for main band (or nuclear) DNA for *Euglena*. As is usual in preparative centrifugations with large amounts of DNA, the satellite (chloroplast-associated) DNA appears as a shoulder on the main band (drops (16-18). Both main band and satellite appear to be labeled. With this assurance that significant incorporation of label from guanine-H^3 is possible, radioautography experiments under identical conditions of labeling were undertaken.

Radioautography of Labeled Cells. Figure 1 shows a representative radioautograph of a light-grown cell of *Euglena* after incubation for 72 hours in guanine-H^3 and removal of the acid-soluble materials. As expected, label is distributed over the cell. If comparable cells are treated with ribonuclease prior to radioautography (fig 2, 3, 4) a significant amount of label is lost. That which remains is consistently found to be localized over the nucleus (fig 2) and in clear clumps over the stained chloroplasts (fig 3, 4). There is no indication of other cytoplasmic clumping of grains. The amount of presumed mitochondrial DNA is about the same as that of chloroplast DNA (5). Since there are more than 10 times as many mitochondria as chloroplasts, the number of grains over the mitochondria should be lower by more than a factor of 10. Thus, this method of labeling selectively permits us to distinguish only DNA label in chloroplasts.

If comparable cells are treated with deoxyribonuclease rather than ribonuclease, (fig 5, 6) there is no significant removal of total radioactivity, consistent with the data presented in table I. There is, however, a selective loss of label from the nucleus as a result of the deoxyribonuclease as might be expected, and the remaining cytoplasmic label is fairly uniformly distributed.

Treatment of the cells with both deoxyribonuclease and ribonuclease, either simultaneously or in

Light micrographs of *Euglena gracilis* var. *bacillaris* labeled with guanine-H^3 and stained with hemotoxylin. Fig. 1. Radioautograph after incorporation of guanine-H^3 for 48 hours and treatment to remove acid soluble materials. The label in both DNA and RNA is distributed over both the nucleus and the cytoplasm of the cell. Fig. 2. Comparable cells to those in 1, treated with ribonuclease prior to radioautography. The remaining label is in the nucleus and is also distributed in clumps over the stained chloroplasts. This picture is focused to show nuclear labeling. Fig. 3, 4. Comparable cells to those in figure 1 treated with ribonuclease prior to radioautography, showing DNA labeling in the nucleus and in clumps over the stained chloroplasts. Fig. 5, 6. Comparable cells to those in figure 1 but treated with deoxyribonuclease prior to radioautography. Most of the label is removed from the nucleus and the remaining cytoplasmic label in RNA is distributed fairly uniformly over the cell. Fig 7, 8. Comparable cells to those in figure 1 treated with deoxyribonuclease and ribonuclease simultaneously. The label is reduced to the level of background.

sequence, reduces the cellular label to background levels (fig 7, 8). No label was removed from control slides incubated in buffer alone. These characteristic labeling patterns were observed in all of the cells in all of the experiments, in both stained and unstained preparations.

This pattern of incorporation and removal of label is completely consistent with the conclusions drawn from previous experiments using other methods (3, 4, 5, 9, 11) that a chloroplast-localized species of DNA exists in *Euglena*.

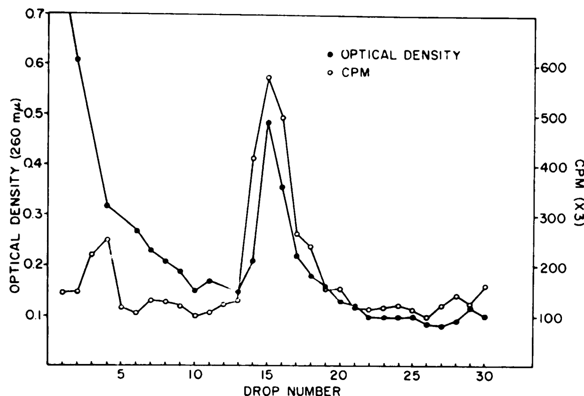

FIG. 9. Density gradient centrifugation of purified DNA isolated from *Euglena*. These cells were incubated in the light for 48 hours in H^3-guanine medium prior to lysis.

Summary

Tritiated guanine or adenine is incorporated into the nucleic acids of *Euglena gracilis* var. *bacilaris* in sufficient quantities to be useful for radioautography. Both RNA and DNA are labeled under these conditions. Radioautography of labeled cells reveals that the radioactivity is distributed over both the nucleus and cytoplasm of the cell. Treatment of the cells with ribonuclease prior to radioautography shows clumps of radioactivity over the nucleus and over the chloroplasts. Treatment with deoxyribonuclease prior to radioautography leaves a fairly uniform RNA labeling pattern over the cell. Treatment with both deoxyribonuclease and ribonuclease removes virtually all of the label in the cells. These results indicate that there is a species of DNA localized in the chloroplasts of the cell. This is consistent with evidence obtained by several other methods.

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