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# Synthesis and Turnover of *Euglena gracilis* Nuclear and Chloroplast Deoxyribonucleic Acid†

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ABSTRACT: The mode of replication of DNA in the chloroplast and in the nucleus of *Euglena gracilis* was investigated by use of a density transfer experiment. The rate of turnover of these DNAs was simultaneously determined by distribution of the density species of nuclear and chloroplast DNA and by uniformly labeling the DNA with [$^{32}$P]$P_i$ and [$^{3}$H]adenine in the presence of the heavy-density label and observing the $^{3}H:^{32}P$ ratio following removal of the [$^{3}$H]adenine and the heavy-density label. Examination of the density profiles for DNA extracted from whole cells and from isolated chloroplasts showed that the nuclear and chloroplast DNA replicate by a semiconservative mechanism. While the nuclear DNA doubles once per cell generation in an exponential culture, chloroplast DNA replication is approximately 1.5 times as fast. Analysis of the $^{3}H:^{32}P$ ratios for nuclear DNA during this density-transfer experiment showed that this DNA was stable. For example, the specific activities of the $^{15}$N strands of the heavy and hybrid DNA were constant prior to the density transfer and following 1.1 and 2.2 cell doublings after the density transfer. Measurement of similar ratios for chloroplast DNA showed decreasing values relative to nuclear DNA during depletion of $^{3}$H pools and after the density transfer, indicative of a half-life of approximately 1.6 generations for this DNA. Since the chloroplast DNA:nuclear DNA ratio is constant in a culture in logarithmic growth, the replication patterns for chloroplast DNA and for nuclear DNA are consistent with the turnover data.

Deoxyribonucleic acid has been clearly demonstrated in chloroplasts and in mitochondria of various organisms. Density-transfer experiments by Chiang and Sueoka (1967) demonstrate semiconservative replication of the chloroplast DNA in *Chlamydomonas reinhardi*. Similar experiments for mitochondrial DNA by Reich and Luck (1966) in *Neurospora crassa* and by Gross and Rabinowitz (1969) in rat liver are interpreted to show semiconservative replication. Temporal separation of the synthesis of nuclear and extranuclear DNA has been observed in synchronously dividing cultures of *Euglena* (Cook, 1966), *Chlamydomonas* (Chiang and Sueoka, 1967), human liver cells (Koch and Stokstad, 1967), *Tetrahymena* (Parsons, 1965), *Physarum polycephalum* (Guttes *et al.*, 1967), and yeast cells (Smith *et al.*, 1968).

The synthesis and breakdown of extranuclear DNA has been studied under various conditions. In regenerating rat liver (Nass, 1967) and during oxygen adaptation in yeast (Rabinowitz *et al.*, 1969), the ratio of mitochondrial to nuclear DNA is greatly increased. In maturing tobacco seedlings (Green and Gordon, 1966) and in normal adult rat liver (Neubert *et al.*, 1968; Schneider and Kuff, 1965; Nass, 1967; Gross *et al.*, 1969), the rate of labeling of extranuclear DNA has been shown to greatly exceed that of nuclear DNA. To maintain a constant ratio between the two classes of DNA, the rapid synthesis of new extranuclear DNA must be balanced by loss of this type of DNA. Such loss has been observed in wheat cells (Hotta *et al.*, 1965) and in various rat tissues (Gross *et al.*, 1969; Neubert *et al.*, 1968). This apparent DNA turnover could be due to mitochondria having a shorter lifetime than the cells within which they exist as "symbionts" (Borst and Kroon, 1969); however, loss and resynthesis of DNA within a persisting mitochondrion has not been excluded.

This paper reports an investigation of the replication and turnover of nuclear and chloroplast DNA in *Euglena gracilis*. Our results indicate that both the nuclear and chloroplast DNA in *E. gracilis* replicate by a semiconservative mechanism. The nuclear DNA is stable while the chloroplast DNA shows turnover equivalent to a half-life of about two cell doublings under our growth conditions.

## Materials and Methods

*Culture Conditions. Euglena gracilis* Klebs, strain Z, was maintained in pure culture at 25° by modification of the autotrophic medium described by Eisenstadt and Brawerman (1967). The $KH_2PO_4$ and $NH_4Cl$ concentrations (either [$^{15}$N]-

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$NH_4Cl$ or [$^{14}$N]$NH_4Cl$) were reduced to 0.15 and 0.30 g per l., respectively, and the medium was supplemented with 2.7 mM sodium citrate. Cell growth was under constant illumination provided by a bank of Gro-Lux lights (Sylvania) in a New Brunswick fermenter containing medium which was equilibrated with 5% $CO_2$ in air. Logarithmic growth was maintained by limiting cell concentrations between $3.7 \times 10^2$ and $3.4 \times 10^5$ cells per ml. Cell counts and viable counts were determined as described previously (Richards *et al.*, 1971). Viable counts were 80–90% of the cell counts.

Cell growth in the fermenter was initiated at a concentration of $3.7 \times 10^2$ cells/ml in 5 l. of medium containing 0.30 g of [$^{15}$N]$NH_4Cl$, 5 mCi of [$^3$H]adenine, and 0.10 mCi of [$^{32}$P]-phosphoric acid per l. of medium. The cells were cultured until a concentration of $3.4 \times 10^5$ cells/ml was attained and then harvested by continuous-flow centrifugation at 25°. The pellet was washed in fresh autotrophic medium at 25° containing 0.30 g of [$^{15}$N]$NH_4Cl$ and 0.08 mCi of [$^{32}$P]phosphoric acid per l. and centrifuged at 3000*g* for 10 min at 25°. The cells were suspended in 14 l. of this medium and cultured from $9.6 \times 10^4$ to $2.1 \times 10^5$ cells per ml. The culture was divided into volumes of 7 l. which were independently and immediately harvested by continuous-flow centrifugation. One pellet was washed in 0.37 M sucrose–0.01 M Tris·HCl–0.05 M EDTA (pH 7.6), centrifuged at 3000*g* for 10 min at 4°, and suspended in four volumes of this same buffer for isolation of chloroplasts. The second pellet was washed in autotrophic medium containing 0.30 g of [$^{14}$N]$NH_4Cl$ and 0.07 mCi of [$^{32}$P]phosphoric acid per l. of medium at 25° and centrifuged at 3000*g* for 10 min at 25°. The cells were suspended in 14 l. of this medium and cultured from a cell concentration of $1.0 \times 10^5$ to $2.2 \times 10^5$ cells per ml. Cell culture (8 l.) was removed, and the cells were collected by continuous-flow centrifugation at 4°. The cells were washed and suspended as before for isolation of chloroplasts. The remaining 6 l. of cell culture was diluted to 14 l. with 8 l. of fresh medium containing 0.07 mCi of [$^{32}$P]phosphoric acid/l. at 25°. The cells were cultured from $7.9 \times 10^4$ to $1.65 \times 10^5$ cells per ml before harvesting by continuous flow centrifugation at 4°. The pellet was washed and suspended as before for chloroplast isolation.

*Isolation of Chloroplasts. Euglena* chloroplasts were isolated by the procedure outlined previously (Manning *et al.*, 1971) through the first sucrose flotation. The pad containing chloroplasts was washed once in 0.37 M sucrose–0.01 M Tris·HCl–0.05 M EDTA (pH 7.6), and once in 0.1 M NaCl–0.01 M Tris·HCl–0.01 M EDTA (pH 7.6). The pellet was suspended in an equal volume of 0.15 M NaCl–0.10 M EDTA–0.05 M Tris·HCl (pH 9.0) and stored at −20°.

*Isolation of DNA.* The total cellular DNA of *E. gracilis* was obtained in a final yield of 60–70% by modifications of the method of Marmur (1961) as described previously (Richards *et al.*, 1971). Chloroplast DNA was obtained from thawed chloroplast suspensions by similar procedures except that dialysis against 5 l. of 0.15 M NaCl–0.015 M sodium citrate–0.5 mM EDTA (pH 8.0), overnight, at 4° was substituted for precipitations by ethanol. *Escherichia coli* bromouracil-labeled hybrid DNA was prepared as described previously (Richards *et al.*, 1971).

*Equilibrium Density Gradient Centrifugation.* Analytical density gradient centrifugation has been described (Richards *et al.*, 1971). Samples of $^{32}$P,$^3$H-labeled chloroplast DNA or of $^{32}$P,$^3$H-labeled whole cell DNA were also centrifuged in the Spinco 40.2 fixed-angle rotor in 3 ml of 0.02 M Tris·HCl (pH 8.0) containing *Clostridium perfringens* DNA ($\rho$ = 1.691 g/cm$^3$) and/or *E. coli* bromouracil-labeled DNA ($\rho$ = 1.754 g/cm$^3$) as density markers. CsCl (3.85 g) was added to this solution, the tubes were filled with mineral oil and were centrifuged at 35,000 rpm for 3 days at 25°. After centrifugation, the tubes were pierced at the bottom with a 22-gauge hypodermic needle, and fractions of 4 drops each were collected, diluted with 0.5 ml of 0.15 M NaCl–0.015 M sodium citrate, and their absorbance at 260 nm and their radioactivity were determined.

For analysis of single-stranded DNA, DNA was denatured and reneutralized by modification of a method described by Davison *et al.* (1964). DNA was denatured in 0.2 N NaOH–2% formaldehyde–0.15 M NaCl–0.015 M sodium citrate for 20 min at room temperature and reneutralized with 1 M $KH_2PO_4$. Marker DNAs and CsCl were added and centrifugation was performed in neutral CsCl density gradients as described above.

*Scintillation Counting.* Fractions from CsCl density gradients were diluted with 0.50 ml of 0.15 M NaCl–0.015 M sodium citrate–0.055 ml of 5 M KOH–0.05 ml of salmon sperm DNA (100 μg), and the fractions were incubated at 37° for 16 hr and then cooled to 4°. Cold 0.61 M trichloroacetic acid (1.43 ml) was added to a final concentration of 0.3 M, and the mixture was kept on ice for 45–60 min. Cold water (2 ml) was added, and the precipitate was collected by vacuum filtration on nitrocellulose filters of 24 mm diameter, Type B6 (Schleicher & Schuell). Filters were washed with 3-ml portions of cold water, transferred to counting vials, and dried at 80°. For radioisotope determination, 10 ml of toluene–liquifluor (4.0 g of 2,5-bis[2-(5-*tert*-butylbenzoxazoly)]-thiophene to 1 l. of toluene) was added to each vial.

*Reagents.* Beef pancreatic ribonuclease A, ribonuclease $T_1$, and *Clostridium perfringens* DNA were obtained from Worthington Biochemical Corp. Salmon sperm DNA and Pronase were obtained from Calbiochem. Optical grade CsCl was purchased from Harshaw Chemical Co. Carrier-free [$^{32}$P]phosphoric acid was purchased from New England Nuclear Corp. [$^3$H]Adenine, labeled in position 2, was obtained from Amersham–Searle Corp. [$^{15}$N]$NH_4Cl$, greater than 95% $^{15}$N enrichment, was obtained from Bio-Rad Laboratories or Teledyne.

## Results

*Experimental Plan.* Our density-transfer experiment is similar to the $^{15}$N-density-labeling experiments of Meselson and Stahl (1958). An outline of the experiment is shown in Figure 1. Specifically, cells were grown exponentially (Figure 2) in an autotrophic medium containing [$^{15}$N]$NH_4Cl$ as the sole nitrogen source and [$^{32}$P]phosphoric acid and [$^3$H]-adenine as radioactive labels for the DNA. After 10 generations of exponential growth, the $^{32}$P in the DNA has equilibrated with the [$^{32}$P]$P_i$ in the medium (J. E. Manning and O. C. Richards, unpublished data). Therefore, after 10 generations of cell growth, the $^{32}$P counts per minute per microgram of DNA are constant. Since $^{32}$P counts are thereafter directly related to micrograms of DNA, these $^{32}$P counts allow us to relate $^3$H counts to absolute amounts of DNA. Then the specific activity of chloroplast DNA can be calculated from the $^3$H:$^{32}$P ratio. Following this equilibration period, the cells were isolated and suspended in autotrophic medium containing [$^{15}$N]$NH_4Cl$ and [$^{32}$P]phosphoric acid but no [$^3$H]adenine. The cells were grown for about one generation to deplete the $^3$H-labeled pools of DNA precursors. The culture was divided into two equal portions, and the

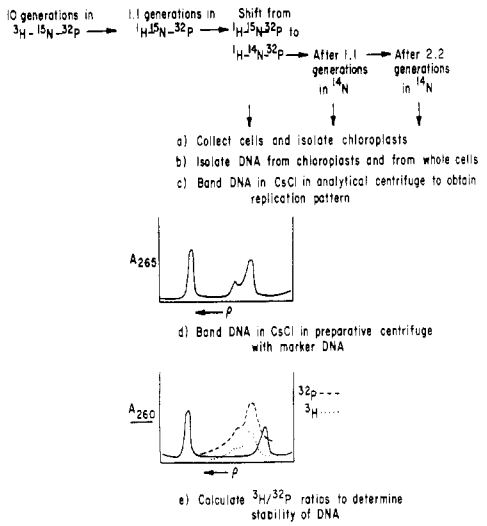


FIGURE 1: Design of density-transfer experiment. The sequence of labeling of *E. gracilis* cells is pictured at the top. Aliquots of the culture were removed at the time of the density transfer and at 1.1 and 2.2 cell doublings following the density transfer. DNA was extracted from whole cells and from isolated chloroplasts (a and b) and banded in a CsCl equilibrium density gradient in the analytical ultracentifuge to determine the pattern of replication (c). The DNA was also centrifuged isopycnically in CsCl with reference DNAs, and fractions were analyzed for absorbance at 260 nm and for radioactivity (d). Isotope ratios were then calculated to determine the stability of the DNA.

cells from both cultures were isolated. The cells from one part were used for the zero-time sample, and those from the second part were suspended in autotrophic medium containing [$^{14}$N]$NH_4Cl$ and [$^{32}$P]phosphoric acid. (A lag in cell division immediately after the density transfer was most likely due to the change in nitrogen isotopes and not due to centrifugation as no lag was observed after removal of [$^3$H]adenine from the medium by centrifugation. See Figure 2.) The cells

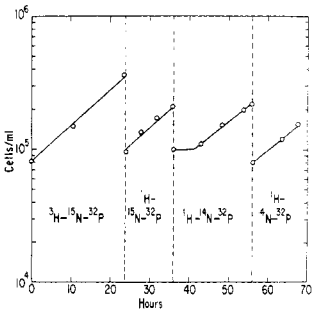


FIGURE 2: Growth curves for *E. gracilis* cells in autotrophic medium. The left portion of the profile shows the last 24 hr of a culture grown exponentially for 10 generations in the presence of 5.0 mCi of [$^3$H]-adenine, 0.3 g of [$^{15}$N]$NH_4Cl$, and 0.1 mCi of [$^{32}$P]$P_i$ per l. of medium. The cells were collected, washed, and grown for 1.1 generations in medium containing 0.3 g of [$^{15}$N]$NH_4Cl$ and 0.08 mCi of [$^{32}$P]$P_i$ per l. of medium but no [$^3$H]adenine. A portion of the culture was removed for the zero-time sample, and the culture remaining was harvested, washed, and suspended in medium containing 0.3 g of [$^{14}$N]$NH_4Cl$ and 0.07 mCi of[$^{32}$P]$P_i$ per l. After 1.1 generations an aliquot of the culture was removed, and the cells were harvested. The culture remaining was diluted with fresh medium containing 0.3 g of [$^{14}$N]$NH_4Cl$ and 0.07 mCi of[$^{32}$P]$P_i$ per l. and harvested after 1.1 cell doublings. Cell counts were determined with a hemocytometer after cells had been killed with one drop of formaldehyde.

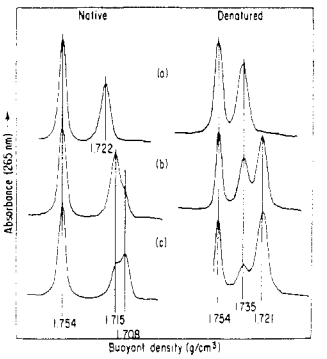


FIGURE 3: Densitometer tracings of photographs after equilibrium density gradient centrifugation of native and denatured DNA. DNA from cells grown 10 generations in medium containing $^{15}N-^{32}P-^3H$ and 1.1 generations in medium containing $^{15}N-^{32}P-^1H$ (a) and from cells harvested 1.1 (b) and 2.2 (c) generations after the density transfer was centrifuged in neutral CsCl equilibrium density gradients in the analytical ultracentrifuge. Denatured DNA was obtained by alkaline denaturation followed by neutralization before centrifugation in CsCl. *E. coli* bromouracil-labeled hybrid DNA ($\rho$ = 1.754 g/cm$^3$) was used as the density marker.

were grown exponentially, and samples were removed at approximately one and two generations.

After removing a small aliquot of whole cells for extraction of nuclear DNA, the chloroplasts were isolated and purified from the three cell samples. The DNA, isolated from the purified chloroplasts and the whole cell samples, was fractionated on the basis of buoyant density by centrifugation in a CsCl equilibrium density gradient in the analytical ultracentrifuge in order to determine the replication pattern of the DNA. The absorption patterns obtained from the analytical ultracentrifuge also indicate DNA turnover. A further determination of DNA turnover was calculated from the $^3H:^{32}P$ ratio ($^3H$ specific activity) of density species of nuclear and chloroplast DNA in preparative CsCl gradients.

*Replication of Nuclear DNA.* The relative proportions of double-stranded nuclear DNA of heavy, hybrid, and light density ($\rho$ = 1.722, 1.715, and 1.708 g per cm$^3$, respectively) can be calculated from the number of cell doublings following a change from $^{15}$N to $^{14}$N in the medium. Thus, in a medium containing [$^{15}$N]$NH_4Cl$, the nuclear DNA will be heavy in density, and one doubling after transfer to a medium containing [$^{14}$N]$NH_4Cl$, the nuclear DNA will be hybrid in density. After two cell doublings in [$^{14}$N]$NH_4Cl$, the ratio of hybrid:light DNA molecules would be 1:1. This expectation requires that the pools of nitrogenous DNA precursors in the cells are quantitatively insignificant and replication is semiconservative.

To test these predictions, the experiment outlined (Figure 1) was performed. The replication patterns for nuclear DNA are illustrated in Figure 3. After 1.1 cell doublings in the $^{14}$N medium, the native nuclear DNA was almost totally hybrid in density with a slight shoulder on the light side corresponding to fully $^{14}$N-labeled DNA (Figure 3b). After 2.2 cell doublings in the $^{14}$N medium, the native nuclear DNA had a major peak corresponding to a density of light DNA and a slightly smaller peak of hybrid DNA (Figure 3c). The densities and ratios of the absorption peaks were, therefore, as expected for semiconservative replication.

Further confirmation of semiconservative replication of the nuclear DNA was obtained by analysis of alkali-denatured DNA in neutral CsCl equilibrium gradients (Figure 3). Only

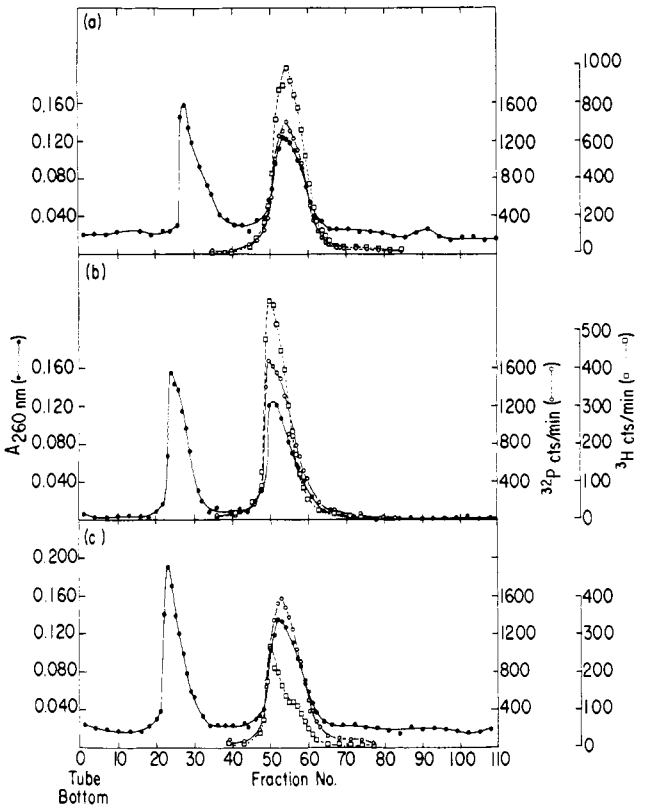


FIGURE 4: Isopycnic centrifugation profiles of native DNA from whole cells. The profiles illustrate the patterns of banding native DNA from cells grown 10 generations in medium containing $^{15}N$–$^{32}P$–$^{3}H$ and 1.1 generations in medium containing $^{15}N$–$^{32}P$-$^{1}H$ (a) and from cells harvested 1.1 (b) and 2.2 (c) generations after the density transfer. Centrifugation was performed in neutral CsCl in the Spinco Model L2 ultracentifuge. Marker DNA was *E. coli* bromouracil-labeled hybrid DNA (fractions 23 to 33). All $^{32}P$ counts were corrected for decay to the start of the experiment. (●) Absorbance at 260 nm; (○) cpm of $^{32}P$; (□) cpm of $^{3}H$.

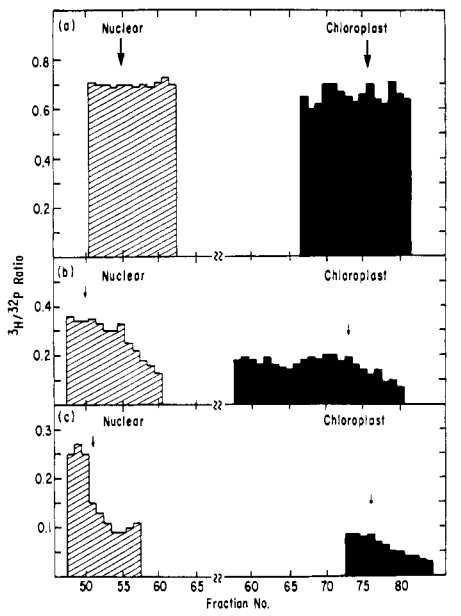


FIGURE 5: Specific activities of native nuclear and chloroplast DNA. $^{32}P$ and $^{3}H$ counts were obtained for the nuclear and chloroplast DNA regions in the neutral CsCl equilibrium gradients described in Figures 4 and 9. Direct comparisons of $^{3}H:^{32}P$ ratios are illustrated for DNA extracted from cells or from chloroplasts obtained prior to the density transfer (a) or 1.1 (b) or 2.2 (c) generations after the density transfer. The heavy arrows in part a indicate the peak fractions in the gradient of fully heavy nuclear and chloroplast DNA. The light arrows in parts b and c show the peak fractions of hybrid DNA.

two density peaks (Figure 3b,c), corresponding to a conserved parental, heavy strand ($\rho$ = 1.735 g/cm$^3$) and a newly synthesized, light strand ($\rho$ = 1.721 g/cm$^3$), were observed for DNA from cells which had grown 1.1 and 2.2 cell doublings in $^{14}N$ medium. The observed density increases of 0.013 g/cm$^3$ are those expected for the formation of single strands from duplex structures of nuclear DNA of heavy and light density. The ratios of the absorption peaks to each other clearly establish that parental, heavy strands are conserved and reaffirm our predictions for semiconservative replication.

*Turnover of Nuclear DNA.* Native DNA from the cell samples described previously was also fractionated in preparative CsCl equilibrium gradients with nonradioactive *E. coli* bromouracil-labeled hybrid DNA ($\rho$ = 1.754 g/cm$^3$) as a density marker. These gradients were fractionated, and the absorbance at 260 nm was determined. Selected fractions were analyzed for radioactivity, and the $^{3}H:^{32}P$ ratio was utilized to give an indication of the stability of parental nuclear DNA strands.

During the growth phase of this experiment, [$^{32}P$]$P_i$ of constant specific activity (accounting for decay of the isotope during the experiment) was maintained. The constancy of the ratio of $^{32}P$ to micrograms of DNA observed in this experiment for all density species of nuclear DNA (Table I) supports our previous experiments showing that DNA-P equilibrates with the medium $P_i$ prior to 10 generations of cell growth. During the equilibration period with $P_i$, the logarithmically growing culture was labeled with [$^{3}H$]adenine in the presence of a $^{15}N$ medium. [$^{3}H$]Adenine was then removed from the medium 1.1 generations before transfer to a $^{14}N$ medium to deplete $^{3}H$-labeled precursors of DNA. Thus, for stable DNA species, $^{3}H$ label would only be present in $^{15}N$ strands of DNA. As a consequence, the $^{3}H:^{32}P$ ratio of the hybrid DNA would show if turnover of the DNA is occurring.

A $^{3}H:^{32}P$ ratio of 0.70 was observed for the fully heavy, native nuclear DNA (Figures 4a and 5a). The $^{3}H$ specific

TABLE 1: The Specific Activity of Nuclear and Chloroplast DNA.[a]

| DNA | Generations in $^{14}N$ | Specific Activity: $^{3}H:^{32}P$ Ratio | Specific Activity: $^{32}P$ cpm/$\mu$g of DNA |
|---|---|---|---|
| Native nuclear | 0 | 0.70 | 568 |
| Native nuclear | 1.1 | 0.35 | 553 |
| Native nuclear | 2.2 | 0.26 | 552 |
| Denatured nuclear | 1.1 | 0.89[b] | 525 |
| Denatured nuclear | 2.2 | 0.81[b] | 542 |
| Native chloroplast | 0 | 0.66 | |
| Native chloroplast | 1.1 | 0.20 | |
| Native chloroplast | 2.2 | 0.085 | |

[a] The $^{3}H:^{32}P$ ratios were calculated from the data in Figures 4, 6, and 9. Culture conditions are described in the legend to Figure 2. [b] Reference is made to the heavy strand.

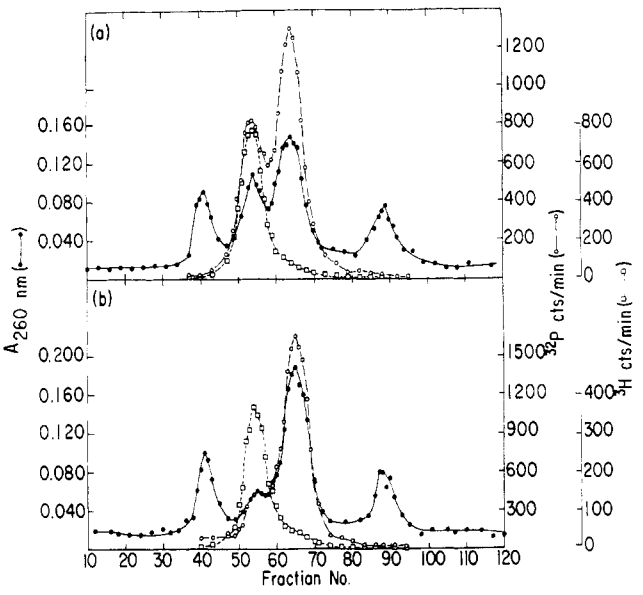


FIGURE 6: Isopycnic centrifugation profiles of denatured DNA from whole cells. DNA was obtained from cells grown 1.1 (a) and 2.2 (b) generations in medium containing $^{14}N$–$^{32}P$–$^{1}H$ after the density transfer (see Figure 2). DNA was denatured, reneutralized, and centrifuged in neutral CsCl in the Spinco Model L2 ultracentrifuge. Marker DNAs were *E. coli* bromouracil-labeled hybrid DNA (fractions 38–45) and *Cl. perfringens* DNA (fractions 85–93) All $^{32}P$ counts were corrected for decay to the start of the experiment. (●) Absorbance at 260 nm; (○) cpm of $^{32}P$; (□) cpm of $^{3}H$.

activity of hybrid DNA would be half this value if the DNA is stable. Such a value is observed for hybrid nuclear DNA after 1.1 generations in $^{14}N$ medium (fractions 48–51 in Figures 4b and 5b, Table I). The lower $^{3}H$:$^{32}P$ ratio for the hybrid region of nuclear DNA after 2.2 generations in $^{14}N$ medium (Figures 4c and 5c) probably reflects the extensive overlap of light DNA into this region of the gradient.

In order to reduce overlap problems and to test for conservation of parental strands of nuclear DNA, denatured

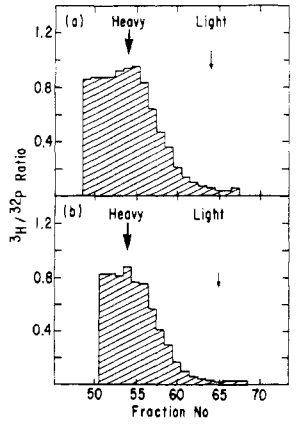


FIGURE 7: Specific activities of denatured DNA from whole cells. $^{32}P$ and $^{3}H$ counts per fraction were obtained for the regions banding the heavy and light strands of denatured nuclear DNA in the neutral CsCl equilibrium gradients described in Figure 6. Ratios of $^{3}H$:$^{32}P$ are shown for DNA extracted from cells grown 1.1 (a) and 2.2 (b) generations after the density shift in medium containing $^{14}N$–$^{32}P$–$^{1}H$. The heavy arrows indicate the peak fractions of the heavy strand and the light arrows indicate the peak fractions of the light strand.

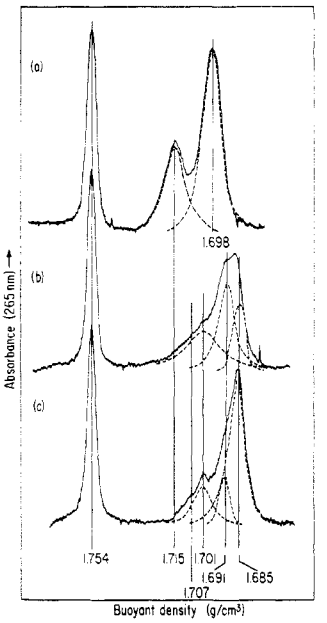


FIGURE 8: Densitometer tracings of photographs after equilibrium density gradient centrifugation of native DNA from chloroplasts. DNA was extracted from chloroplasts purified from cells obtained prior to the density transfer (a) (see Figure 2) and from cells grown 1.1 generations (b) and 2.2 generations (c) after the density transfer in medium containing $^{14}N$–$^{32}P$–$^{1}H$. The DNA was centrifuged in neutral CsCl equilibrium density gradients in the analytical ultracentrifuge, and tracings of the absorption patterns were made by a Joyce-Loebl recording densitometer (solid line). The densitometer tracings were resolved into various density species of chloroplast DNA by a Dupont 310 curve resolver (dashed line). *E. coli* bromouracil-labeled hybrid DNA ($\rho$ = 1.754 g/cm$^3$) was used as the density marker.

DNA was fractionated in neutral CsCl equilibrium density gradients. Figure 6 illustrates the density profiles of this DNA isolated from cells grown 1.1 and 2.2 generations in $^{14}N$ medium after the density transfer. *E. coli* bromouracil-labeled hybrid DNA and *Clostridium perfringens* DNA were used as density markers. The two peaks of nuclear DNA represent heavy ($\rho$ = 1.735 g/cm$^3$) and light ($\rho$ = 1.721 g/cm$^3$) strands. Practically all $^{3}H$ label is found in heavy strands for both these samples. Furthermore, the $^{3}H$ specific activity of the heavy strands is about 0.85 (Figure 7), a value similar to that observed for native, heavy nuclear DNA. Analysis of $^{3}H$:$^{32}P$ ratios establish that nuclear DNA is stable during the duration of this experiment. Thus, the results obtained for this DNA can be used as an internal standard for the determination of turnover of chloroplast DNA.

*Replication of Chloroplast DNA.* The mode of replication for chloroplast DNA of *E. gracilis* was determined using the methods previously described for the nuclear DNA. Purified chloroplast DNA obtained from cells harvested immediately prior to the density transfer showed a major DNA species at a density of 1.698 g/cm$^3$ (Figure 8a), representative of heavy chloroplast DNA, and a minor DNA species at a density of 1.715 g/cm$^3$. This minor DNA has been shown by Stutz and Vandrey (1971) to be a fraction of chloroplast DNA enriched in chloroplast rRNA cistrons. The curves in this figure were resolved into DNA bands using a Dupont 310 curve resolver, and the data are summarized in Table II. For all densitometer traces, the minor DNA was found to comprise 34–37% of the total chloroplast DNA. After 1.1 cell doublings in the $^{14}N$ medium, the major chloro-

TABLE II: Resolution of Main-Band Chloroplast DNA by Dupont Curve Resolver.

| Generations in $^{14}N$ | DNA Species | Buoyant Density (g/cm$^3$) | % of Main-Band Chloroplast DNA | % of Total ChloroplastDNA |
|---|---|---|---|---|
| 0 | Heavy | 1.698 | 100 | 65 |
| 1.1 | Hybrid | 1.691 | 63 | 40 |
| 1.1 | Light | 1.685 | 37 | 23 |
| 2.2 | Hybrid | 1.691 | 20 | 13 |
| 2.2 | Light | 1.685 | 80 | 53 |

plast DNA species appears as two bands at densities of 1.691 and 1.685 g per cm$^3$ (Figure 8b), representative of hybrid and light chloroplast DNA, in the ratio of 2:1. Also apparent are shoulders at densities of 1.707 and 1.701 g per cm$^3$. These density values are consistent with hybrid and light DNA species of the minor DNA. (Resolution of the light species of the minor DNA is shown in Figure 8b.) After 2.2 cell doublings in the $^{14}N$ medium, the major chloroplast DNA had buoyant densities of 1.691 and 1.685 (Figure 8c) in the ratio of 1 part hybrid species to 4 parts light species (Table II). Similarly, the minor DNA was predominantly light. Resolution of the minor species of chloroplast DNA after 1.1 and 2.2 cell doublings in the curve resolver was not adequate to determine the rate of its replication; however, only heavy, hybrid, and light species of this minor DNA were observed throughout this experiment. Thus, semiconservative replication, both of the major chloroplast DNA species and of the minor DNA in these chloroplast preparations, is indicated.

*Turnover of Chloroplast DNA.* In a logarithmic culture of *E. gracilis* the ratio of nuclear DNA:chloroplast DNA is constant. As we have shown, the nuclear DNA replicates once per cell division and is stable. In contrast, the chloroplast DNA replicates 1.5 times/cell division (Figure 8). Since the ratio of nuclear DNA:chloroplast DNA is constant, one-third of the total chloroplast DNA in each cell generation must be degraded.

To further substantiate the turnover indicated by the density patterns from analytical ultracentrifugation, we have independently measured turnover by comparison of the $^3H$ specific activity of the heavy and hybrid species. Native DNA was extracted from chloroplasts that were isolated from cells harvested immediately prior to the density transfer and at 1.1 and 2.2 cell doublings following the density transfer. This DNA was fractionated in preparative CsCl equilibrium gradients with *E. coli* bromouracil-labeled hybrid DNA and *Cl. perfringens* DNA as density markers (Figure 9). The gradients were analyzed for absorbance and radioactivity as described for nuclear DNA, and the $^3H$ specific activity of the heavy and hybrid DNA was calculated (Figure 5). In evaluations of $^3H$ specific activity, we wish to emphasize that identical absolute differences in specific activity at low and high $^3H$:$^{32}P$ ratios have unequal weight.

Since the $^3H$ label is in adenine and chloroplast DNA has 36.5% adenine as compared to 24% adenine in the nuclear DNA (Ray and Hanawalt, 1964; Edelman *et al.*, 1965, 1964; Brawerman and Eisenstadt, 1964), chloroplast DNA would

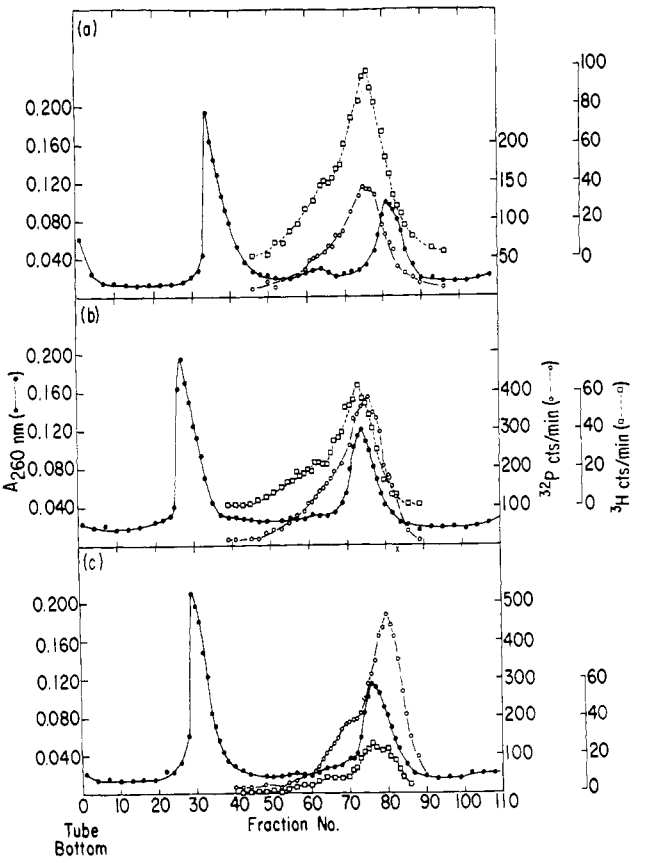


FIGURE 9: Isopycnic centrifugation profiles of native DNA from isolated chloroplasts. Chloroplasts were isolated from cells obtained prior to the density transfer (a) (see Figure 2) and from cells grown for 1.1 (b) and 2.2 (c) generations after the density transfer in medium containing $^{14}N$–$^{32}P$–$^1H$. DNA was extracted from these chloroplast preparations, and the DNA was centrifuged preparatively in neutral CsCl with *E. coli* bromouracil-labeled hybrid DNA (fraction 25–35) and *Cl. perfringens* DNA (fractions 70–85) as marker DNAs. All $^{32}P$ counts were corrected for decay to the start of the experiment. (●) Absorbance at 260 nm; (○) cpm of $^{32}P$; (□) cpm of $^3H$.

be expected to have 1.52 times more $^3H$ label per microgram of DNA than the nuclear DNA has. In a previous density transfer experiment, prior to depletion of $^3H$ pools, we observed 1.46 times more $^3H$ label per microgram of DNA in chloroplast DNA than in nuclear DNA. (Similar experiments using $^{32}P$ labeling show no increased specific activity of chloroplast DNA, provided that DNA-P has equilibrated with medium $P_i$.) This expectation would result in a $^3H$ specific activity of 1.06 for chloroplast DNA after radioisotope equilibration in our experiment. However, the $^3H$ label was removed from the medium 1.1 generations before the density transfer, and the $^3H$ specific activities observed for fully heavy nuclear and chloroplast DNA were 0.70 and 0.66, respectively (Figure 5a, Table I), in the zero-time sample, indicating turnover of the chloroplast DNA is occurring or pools of DNA precursors containing adenine are more extensive in the nucleus than in the chloroplast. Further evidence for chloroplast DNA turnover is indicated after the density transfer. A $^3H$ specific activity of 0.20 is observed in the hybrid region for chloroplast DNA at 1.1 cell doublings (fractions 69–71, Figures 5b and 9b). Since a specific activity of 0.33 is expected for stable hybrid DNA, turnover of DNA is again indicated. It should be emphasized that both strands of the hybrid DNA contain $^{32}P$ as well as both strands of

the light DNA. Therefore, the $^{32}P$ counts in the hybrid region in Figure 9b represent not only light DNA but also hybrid DNA. In the calculation of the specific activity of the hybrid DNA, we have minimized the contribution of $^{32}P$ counts from light DNA by selecting fractions on the heavy side of the hybrid DNA peak. A ratio of 0.085 is observed at 2.2 cell doublings (Figure 5c), but this value is underestimated because of the overlap of light DNA into the hybrid region. From the specific activities for chloroplast DNA observed prior to the density transfer and at 1.1 cell doublings, the half-life (Chase and Rabinowitz, 1962) of chloroplast DNA is 1.6 cell doublings.

## Discussion

The mode of replication of the nuclear DNA clearly follows that predicted for a semiconservative mechanism. This was shown by the density patterns obtained for both the duplex DNA and the denatured DNA. The nuclear DNA had density species representative of hybrid and light nuclear DNA with no observable DNA species of intermediate density following 1.1 and 2.2 cell doublings after the density transfer. Furthermore, the ratio of hybrid:light DNA was that predicted for DNA replicating once per cell doubling by a semiconservative mechanism. In the density patterns of the denatured DNA, no bands of density other than the light and heavy strands of the nuclear DNA were present, reaffirming the conservation of DNA strands.

The replication pattern of chloroplast DNA follows a semiconservative mechanism, but replication is approximately 1.5 times as fast as that for nuclear DNA. Heavy, hybrid, and light species of chloroplast DNA are observed. Due to the base bias between strands of *E. gracilis* chloroplast DNA (Stutz and Rawson, 1970), examination of denatured chloroplast DNA is not feasible in a density-transfer experiment because of the appearance of multiple density species which cannot be resolved. A conservative replication would only give heavy and light DNA; thus, this replication scheme is eliminated. Any type of dispersive mechanism requires rapid disappearance of hybrid structures. We observe hybrid DNA after one and two generations in the proportion expected for 1.5 semiconservative replications per generation. Therefore, replication of chloroplast DNA is consistent with a semiconservative scheme.

The specific activities of both the native and denatured nuclear DNA following 1.1 and 2.2 cell doublings indicate that this DNA is stable under these growth conditions. After 1.1 cell doublings in $[^{14}N]NH_4Cl$, the $^{3}H:^{32}P$ ratio of the hybrid DNA is half that of the heavy DNA, as would be expected for a stable DNA species. The decrease in the specific activity of the hybrid DNA to one-third that of heavy DNA after 2.2 cell doublings probably reflects the overlap of the light DNA into the hybrid DNA region. Computations for turnover of native DNA are best calculated from samples having mostly hybrid species, for example, from the 1.1 generation sample. The specific activity of the heavy strand of the hybrid duplex DNA is identical following 1.1 and 2.2 cell doublings, supporting the interpretation of a stable DNA species. Since we are measuring disappearance of label from $^{15}N$-labeled DNA strands, contributions of $^{3}H$ to DNA precursor pools from RNA, protein, or lipid turnover are not important ($^{3}H$ would be predominantly incorporated into the $^{14}N$ strand).

Since the chloroplast DNA:nuclear DNA ratio is constant in a culture of *E. gracilis* in logarithmic growth, chloroplast DNA which doubles 1.5 times as fast as the cell doubles must exhibit the loss of 1 equiv of itself/cell generation. Measurement of the turnover of chloroplast DNA by examination of the $^{3}H$ specific activity permitted a further estimation of the half-life of this DNA (Chase and Rabinowitz, 1962); a half-life of approximately 1.6 generations was obtained and is in good agreement with the replication pattern. This is the first good evidence for chloroplast DNA turnover, especially for cells in logarithmic culture. In these experiments ratios of radioisotopes were used as a measure of specific activity due to the minute quantities of chloroplast DNA which could be obtained. The suitability of such an approach is supported by these results.

The mechanism of chloroplast DNA turnover cannot be defined by these experiments. Turnover may be due to exchange of pieces of DNA or complete breakdown of molecules. Our experiments do not support excision and repair of short segments of DNA within whole molecules for the total explanation of the turnover, as we observe the formation of discrete density species (hybrid and light DNA) after the density shift, even after two generations. However, we cannot exclude this process as a minor factor contributing toward some of the turnover observed. Complete breakdown of chloroplast DNA molecules, *e.g.*, in a persisting chloroplast or due to organelle turnover, as a possible cause of turnover does not violate a semiconservative replication.

A minor DNA species has recently been described by Stutz and Vandrey (1971) in the DNA isolated from the chloroplast fraction of *E. gracilis*. Hybridization experiments have shown that this DNA is enriched for rRNA cistrons. This DNA has been previously observed in DNA prepared from chloroplasts (Manning *et al.*, 1971). More recent experiments (O. C. Richards, 1971, unpublished data) have shown that the minor DNA comprises a larger portion of the chloroplast DNA in logarithmic cultures than in stationary cultures. This explains the increased amount of minor DNA found in these experiments compared to the previous report (Manning *et al.*, 1971). Its buoyant density in $^{14}N$ medium is 1.701 and in $^{15}N$ medium is 1.715 g/cm$^3$. Following the density shift, DNA bands appear at densities of 1.707 and 1.701 g per cm$^3$ which are densities expected for hybrid and light molecules of this DNA. The observation that a species of this DNA of hybrid density appears following 1.1 cell doublings indicates that this DNA is a double-stranded molecule. The ratio of hybrid:light DNA following 1.1 and 2.2 cell doublings could not be evaluated in these experiments; however, the appearance of distinct heavy, hybrid, and light species suggests that replication of this DNA is by a semiconservative mechanism.

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# Divalent Cations in Transfer Ribonucleic Acid and Aminoacyl Transfer Ribonucleic Acid Synthetase Function and Structure†

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ABSTRACT: $Mn^{2+}$ serves as well as $Mg^{2+}$ to support the function of the isoleucyl-tRNA synthetase ($tRNA^{Ile}$) system of *Escherichia coli* B; therefore $^{54}Mn^{2+}$ has been used to determine the role of divalent ions in this process. These ions are easily removed from tRNA, isoleucyl-tRNA synthetase, and isoleucyl-tRNA synthetase (AMP-Ile), and the purified system containing deionized $tRNA^{Ile}$ and isoelucyl-tRNA synthetase (AMP-Ile) is still capable of aminoacylation, even in 0.2 M $Na_2EDTA$. We conclude that neither recognition of tRNA, nor stability of isoleucyl-tRNA synthetase (AMP-Ile), nor transfer of isoleucine from isoleucyl-tRNA synthetase (AMP-Ile) to tRNA requires stoichiometric or even catalytic participation of a divalent metal. Thus, in this system, divalent cations seem to be required only in the synthesis of isoleucyl-tRNA synthetase (AMP-Ile). Divalent metal ion(s) therefore cannot be a part of the static or dynamic structure of $tRNA^{Ile}$ which is required for recognition and aminoacylation.

The pronounced effects of divalent cations ($M^{2+}$) on tRNA structure (Millar and Steiner, 1966) have long been known (Penniston and Doty, 1963). The implications for tRNA function of the large increase in order and stability attained (Reeves *et al.*, 1970; Rosenfeld *et al.*, 1970) when divalent cations are bound to tRNA (Cohn *et al.*, 1969) are less clear, however. It appears that certain tRNAs may be trapped in an inactive conformation by depriving them of these ions (Gartland and Sueoka, 1966; Fresco *et al.*, 1966), but divalent cations, though they seem to catalyze a transformation from the "denatured" or inactive, to "native" and active disposition, may not be required for maintenance of the active state, once attained (Fresco *et al.*, 1966; Ishida and Sueoka, 1968). This behavior, however, is not general: the behavior of most tRNAs is unaffected by the treatments which affect the "denaturable" class. Further, divalent cations are persistent contaminants of many reagents used in studying

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