EXPRESSION OF THE MITOCHONDRIAL GENOME IN HeLa CELLS

XV. Effect of Inhibition of Mitochondrial Protein Synthesis on Mitochondrial Formation

BRIAN STORRIE and GIUSEPPE ATTARDI

From the Division of Biology, California Institute of Technology, Pasadena, California 91109. Dr. Storrie's present address is the Department of Biophysics and Genetics, University of Colorado Medical Center, Denver, Colorado 80220.

ABSTRACT

The effect of selective inhibition of mitochondrial protein synthesis by chloramphenicol at 40 or 200 μg/ml on the formation of mitochondria in HeLa cells was investigated. HeLa cells, under the conditions used in the present work, grow at a decreasing rate for at least four cell generations in the presence of 40 μg/ml chloramphenicol, and for two generations in the presence of 200 μg/ml chloramphenicol. The progressive cell growth inhibition which begins after 2 days of exposure of the cells to 40 μg/ml chloramphenicol is immediately or gradually reversible, upon removal of the drug, for periods up to at least 8 days of treatment, though there is a progressive loss of cloning efficiency. In cells which have been treated for 6–7 days with 40 or 200 μg/ml of chloramphenicol, mitochondrial protein synthesis occurs at a normal or near-normal rate 1 h after removal of the drug. Mitochondria increase normally in number and show a normal size and amount of cristae in the presence of either concentration of drug. However, in 4–5% of the mitochondrial profiles the cristae appear to be arranged in unusual, circular, looped or whorled configuration.

INTRODUCTION

In HeLa cells exposed to a concentration of chloramphenicol (40 μg/ml) sufficient to inhibit overall mitochondrial protein synthesis, mitochondrial RNA and DNA synthesis continues at a normal or near-normal rate for as many as 2.5 cell generations, and mitochondrial DNA and the soluble mitochondrial enzyme, malate dehydrogenase, accumulate normally (Storrie and Attardi, 1972). These observations indicate either that HeLa cell mitochondria are increasing normally in number in the absence of mitochondrial protein synthesis, or that the amount per mitochondrion of DNA, malate dehydrogenase, and presumably other cytoplasmically synthesized mitochondrial enzymes, and possibly RNA, is increasing with a likely increase in size of the organelle. The persistence of mitochondria in "petite" yeast mutants, in which mitochondrial protein synthesis does not occur (Linnane and Haslam, 1970), suggests that the first alternative is the correct one.

In the present work, the effect of selective inhibition of mitochondrial protein synthesis by chloramphenicol on the formation of the mito-

chondrial protein synthetic machinery, on the assembly of the inner mitochondrial membrane, and on the formation of mitochondria has been investigated in HeLa cells. Besides the previously used concentration of 40 μg/ml of chloramphenicol, a much higher concentration (200 μg/ml) was also used because of the possible occurrence of a fraction of mitochondrial protein synthesis particularly resistant to the drug, as has been reported for bacteria (Lark and Lark, 1966; Levine and Sinsheimer, 1969). Treatment of the cells with this drug at 40 or 200 μg/ml for periods of up to 7 days has been found to have little or no effect on the rate of formation of mitochondria and on cell capacity for mitochondrial protein synthesis, assayed 1 h after removal of the drug. The progressive inhibition of cell growth, which occurs after more than 2 days of exposure to 40 μg/ml chloramphenicol, is immediately or gradually reversible, upon drug removal, for periods of at least 8 days of treatment.

MATERIALS AND METHODS

Cells, Method of Growth, Treatment of Cells with Chloramphenicol, and Assay of Cloning Efficiency

HeLa cells were grown in suspension in modified Eagle's medium (Levintow and Darnell, 1960) with 5% calf serum, as described (Amaldi and Attardi, 1968), with a generation time of about 20 h. The cells used were free of any detectable contamination by *Mycoplasma*. Cells were counted with a hemacytometer; each count is the average of three separate samplings of a culture. Cell concentrations never exceeded 6×10^5 cells/ml.

Cells were treated with chloramphenicol at a concentration of either 40 or 200 μg/ml to inhibit mitochondrial protein synthesis. Either concentration is sufficient to inhibit more than 90% of the amino acid-incorporating activity which is insensitive to emetine inhibition of cytoplasmic protein synthesis (Storrie and Attardi, 1972). HeLa cell cultures maintained for long periods of time in the presence of chloramphenicol were diluted with fresh chloramphenicol containing medium every 2–3 days.

Cells to be tested for reversibility of chloramphenicol inhibition of cell growth were pretreated for varying times with 40 μg/ml chloramphenicol, centrifuged down, washed twice with chloramphenicol-free, modified Eagle's medium with 5% calf serum, and resuspended in the same medium at $5–8 \times 10^4$ cells/ml. To assay the cloning efficiency of chloramphenicol-pretreated cells, duplicate samples of 1,000 cells from each washed, chloramphenicol-free culture and similarly treated control cultures were plated on 60 × 15 mm Falcon Plastics Petri dishes with 2 mm grid (Falcon Plastics, Oxnard, Calif.) and grown for 3 days in modified Eagle's medium with 5% calf serum. Clones were scored after staining of the clones sequentially with May-Grünwald and Giemsa stains. The cloning efficiency of control cells was about 33%.

Assay of Mitochondrial Protein Synthesis

LABELING CONDITIONS: To test the effect of prolonged cell growth in the presence of chloramphenicol on the functionality of the mitochondrial protein-synthesizing machinery, HeLa cells grown for varying periods of time in the presence of 40 or 200 μg/ml of the drug were centrifuged down, washed twice or, respectively, three times in modified Eagle's medium with 5% dialyzed calf serum, and resuspended in the same warm medium at a concentration of $8–10 \times 10^5$ cells/ml; after 55 min at 37°C, the cells were treated with 200 μg/ml emetine for 5 min to inhibit (essentially completely) cytoplasmic protein synthesis, and then exposed to L-[4,5-^{3}H]leucine (58 mCi/μmol, 1.3 μCi/ml) for 30 min in the presence of emetine. Under these conditions, more than 90% of the amino acid incorporation is sensitive to chloramphenicol (Perlman and Penman, 1970; Ojala and Attardi, 1972; Storrie and Attardi, 1972). To provide an internal standard to correct for differences in extent of cell homogenization, the cell cultures pulse labeled with L-[4,5-^{3}H]leucine were mixed with a constant amount ($\sim 4.5 \times 10^7$) of non-drug-treated cells labeled for 1 day with [2-^{14}C]uridine (62 μCi/μmol, 0.001 μCi/ml). (In HeLa cell crude mitochondrial preparations, the amount of ribosomal RNA from contaminating endoplasmic reticulum-bound ribosomes has been shown to be proportional to the amount of mitochondria, as estimated from the cytochrome oxidase activity [Pica-Mattoccia and Attardi, 1971].) Normalization of the [^{3}H]leucine incorporation data was performed by dividing them by the corresponding [2-^{14}C]uridine labeling values.

PREPARATION OF MITOCHONDRIA AND ANALYSIS OF RADIOACTIVITY: A crude mitochondrial fraction containing the bulk of HeLa cell mitochondria and rough endoplasmic reticulum was prepared by differential centrifugation (Attardi et al., 1969). In order to analyze the incorporation of [^{3}H]leucine into mitochondrial protein, samples of the washed crude mitochondrial fraction were resuspended in 1.0 N NaOH for 1 h at 37°C to hydrolyze the RNA; after neutralization with 1 N HCl, the samples were either precipitated directly with 5% trichloroacetic acid, or solubilized with 1% sodium dodecyl sulfate and then precipitated with 15% trichloroacetic acid. The precipitates were collected on Millipore membranes

(Millipore Corp., Bedford, Mass.) and counted in a scintillation counter. No consistent difference in the amount of acid-precipitable ^{3}H counts per minute was found by using the two methods of acid precipitation; therefore, the data of [^{3}H]leucine radioactivity precipitated in the presence or absence of sodium dodecyl sulfate, after correction for variation in cell homogenization on the basis of long-term [2-^{14}C]uridine radioactivity (which is incorporated largely into cytoplasmic ribosomal RNA), have been averaged here.

Preparation of Cells for Electron Microscopy and Quantitative Analysis of Electron Micrographs

Cells grown for varying periods in the presence or absence of 40 or 200 μg/ml chloramphenicol were centrifuged down, washed twice in modified Eagle's medium, and fixed in suspension for 1 h at 2°C in a freshly prepared solution containing 2% glutaraldehyde and 1% formaldehyde in 0.1 M cacodylate buffer, pH 7.4, 0.125 mg/ml $CaCl_2$, and 0.25 mg/ml trinitrocresol. The fixed cells were packed into a tight pellet by centrifugation at 4,000 rpm in the International 269 rotor (International Equipment Co., Needham Heights, Mass.) for 15 min at 2°C and the pellet was broken into 1-mm-thick blocks, which were rinsed with 0.1 M cacodylate buffer, pH 7.4, for 1 h at 2°C, and then postfixed with 1% osmium tetroxide in 0.1 M collidine buffer, pH 7.4, for 1 h at room temperature. The blocks were subsequently rinsed three times in 0.05 M maleate buffer, pH 5.1, stained for 1 h at room temperature in 2% uranyl acetate in 0.05 maleate buffer, pH 6.0, rinsed three more times with 0.05 M maleate buffer, pH 5.1, dehydrated in a graded series of alcohol solutions, and washed twice for 15 min each in propylene oxide. The cell blocks were then embedded in Epon 812 introduced through graded concentrations of propylene oxide. Gold sections (~100 nm thick) were cut and stained for 3 min with lead citrate (Venable and Coggeshall, 1965). Electron micrographs were taken with a Philips 200 electron microscope set at 60 kV.

To estimate the number of mitochondria per unit of cross-sectional area of cytoplasm before and after chloramphenicol treatment, micrographs of the HeLa cell cross sections were printed to give a × 15,000 overall magnification of the cells. The contours of the cell body, the nucleus, and individual mitochondria (scored as organelle profiles having a minimum of one crista) in the cross section of each cell were drawn on tracing paper. The cross-sectional area of the cytoplasm and that of all mitochondria in each cell section were then determined from the weight of the tracing paper.

To determine the average ratio of cristae length to outer membrane length and the average ratio of cristae length to mitochondrial cross-sectional area, micrographs of portions of individual HeLa cells containing 10–20 mitochondria were printed to give × 32,000 overall magnification. The outer mitochondrial membranes and cristae were traced, and the overall lengths of the outer membranes and, respectively, of the cristae in the sample of mitochondria from each cell were measured with a map measurer or a ruler, and the overall cross-sectional area of the organelles was determined from the weight of the tracing paper.

Histochemical Localization of Cytochrome c Oxidase Activity

Cytochrome *c* oxidase activity was localized along the inner mitochondrial membrane by the 3,3′-diaminobenzidine tetrahydrochloride (DAB) method of Seligman et al. (1968), as modified and adapted to cells cultivated in vitro (Posakony et al., manuscript in preparation). In brief, the modified procedure involved fixation of the cells in 3% formaldehyde in 0.44 M sucrose, 0.05 M cacodylate buffer, pH 7.4, containing 1 mg/ml $CaCl_2$, washing of the cells in sucrose-cacodylate-EDTA buffer, and incubation in 0.5 mg/ml DAB solution in 0.44 M sucrose, 0.05 M Tris buffer, pH 7.4 (25°C), 2×10^{-5} M EDTA. Parallel control incubations with the cytochrome oxidase inhibitor, potassium cyanide, added to a concentration of 0.001 M 15 min before the addition of DAB, were performed. After the cells were washed in sucrose-Tris buffer, they were postfixed in suspension with 1% osmium tetroxide, 0.1 M collidine buffer, pH 7.4 (25°C) for 1 h at room temperature, and centrifuged down at 4,000 rpm at room temperature in the International 269 rotor for 15 min to give a firm pellet. The pellets were broken into blocks, dehydrated in a graded series of ethanol concentrations starting at 50%, and embedded for electron microscopy as above. Gold sections were cut and observed in the electron microscope before and after treatment for 30 s with lead citrate.

RESULTS

Effects of Chloramphenicol on Cell Growth and Their Reversibility

Fig. 1 shows representative growth curves of HeLa cell cultures maintained in modified Eagle's medium with 5% calf serum in the presence of either 40 or 200 μg/ml chloramphenicol. Cells grown under these conditions in the presence of 40 μg/ml chloramphenicol multiply

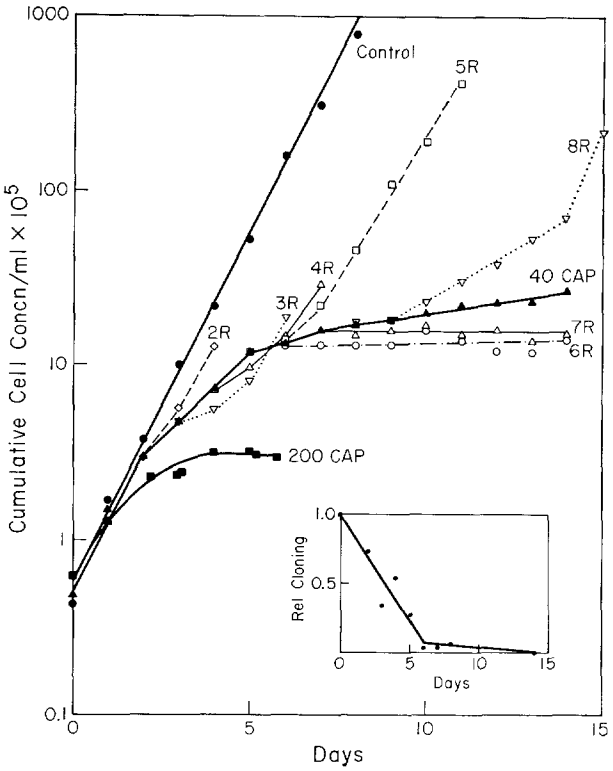

FIGURE 1 Growth of HeLa cells in the presence of 40 or 200 μg/ml chloramphenicol (CAP) and after removal of the drug. Parallel cultures of HeLa cells were grown in the absence or presence of either 40 μg/ml (40 CAP) or 200 μg/ml (200 CAP) chloramphenicol. After 2 or more days of exposure to 40 μg/ml of the drug, a portion of the treated culture was washed and transferred to chloramphenicol-free medium (2R-8R). Both the original and the transferred cultures were diluted to 5–10 × 10^4 cells/ml with fresh medium every 2–3 days. ●——●, cumulative cell concentration per milliliter in absence of chloramphenicol; ▲——▲, cumulative cell concentration per milliliter in presence of 40 μg/ml chloramphenicol (40 CAP); ■——■, cumulative cell concentration per milliliter in presence of 200 μg/ml chloramphenicol (200 CAP); Open symbols (2R,3R–8R), cumulative cell concentration per milliliter of a culture washed and transferred after 2–8 days of exposure to chloramphenicol at 40 μg/ml. *Insert*: duplicate samples of each washed, chloramphenicol-free culture at the time of transfer, or of a similarly treated control culture, were plated on plastic Petri dishes; •——•, relative cloning efficiency of washed, chloramphenicol-free cultures after different lengths of drug treatment, and of control cultures.

with a normal 20 h generation time for 2 days, and over the next 3 days increase in number with an apparent 36 h generation time, so that they go through about four cell generations of growth in the first 5 days of exposure to the drug. After 7 days of treatment with chloramphenicol, the growth of the culture apparently stabilizes at a rate corresponding to one generation in 270 h (~11 days), i.e., 7% of the control growth rate. Cells maintained in the presence of 200 μg/ml chloramphenicol grow at a progressively decreasing rate until, after 4 days or about two generations of growth, the culture enters a stationary phase.

Fig. 1 also shows the growth of portions of the 40 μg/ml chloramphenicol-treated culture after removal of the drug. When transferred to chloramphenicol-free medium, cells exposed to the drug for 2 days continued to grow at a normal rate. After exposure to chloramphenicol for 3–5 days, in this experiment, on the contrary, the cells resumed a normal or near-normal growth rate only gradually, over a period of 1–2 days. Cells transferred to chloramphenicol-free medium after 6–8 days either showed no growth at all for 1 wk or more (6R, 7R), or resumed a normal growth rate only after 6 days of gradual recovery (8R). The reason for the differences in recovery of the cultures transferred to

chloramphenicol-free medium after 6–8 days is not known. A culture transferred to chloramphenicol-free medium after 14 days showed no indication of recovery after 1 wk.

It should be noted that the results of transfer of chloramphenicol-treated cells to drug-free medium have been somewhat variable. In some experiments, cultures have resumed a normal or near-normal growth rate immediately upon transfer to drug-free medium after periods of growth in the presence of 40 μg/ml chloramphenicol up to 5 days. In no case have cells failed to recover a normal growth rate within 1–2 days after transfer, if this was made after 5 or fewer days in chloramphenicol-containing medium. On the contrary, once the cells had reached a nearly stationary growth phase after 6 or more days of exposure to chloramphenicol, several days in drug-free medium were found in all experiments to be required for recovery, if this occurred at all. A possible source of the variability observed between experiments may be the different lots of calf serum used.

In the experiment shown in Fig. 1, the cloning efficiency on solid substrate of HeLa cells transferred to chloramphenicol-free medium decreased with increasing length of exposure to 40 μg/ml chloramphenicol (insert to Fig. 1). The cloning efficiency of drug-treated cells relative to that of control cells was ~75% after 2 days of drug treatment, ~30% after 5 days, ~5% after 6–8 days, and less than 1% after 14 days. The clone size was normal for drug-treated cells transferred after 2–5 days of drug exposure. After 6 or more days of drug exposure, the clone size was 25–50% of control.

Effect of Chloramphenicol Pretreatment on the Rate of Mitochondrial Protein Synthesis

The immediate to rapid recovery of growth rate and the normal clone size of cells treated with 40 μg/ml chloramphenicol for 2–5 days, after removal of the drug, suggested a corresponding rapid resumption of mitochondrial protein synthesis upon removal of chloramphenicol. To test this possibility, HeLa cell cultures treated for varying times with 40 or 200 μg/ml chloramphenicol were transferred to drug-free medium, and after 1 h exposed to a 30 min [^{3}H]leucine pulse in the presence of 200 μg/ml emetine; the incorporation of label into the mitochondrial fraction was then analyzed. Emetine-insensitive [^{3}H]leucine labeling of the mitochondrial fraction can be considered equivalent to mitochondrial protein synthesis, since more than 90% of it is sensitive to inhibitors of mitochondrial protein synthesis, such as chloramphenicol (Perlman and Penman, 1970; Ojala and Attardi, 1972; Storrie and Attardi, 1972). In control experiments, it was found that the effect of a 15 min treatment of HeLa cells with 40 μg/ml chloramphenicol on the rate of mitochondrial protein synthesis, as measured in a 30 min [^{3}H] leucine pulse in the presence of emetine, was completely reversible within 30 min of growth in chloramphenicol-free medium.

Fig. 2 shows the relative incorporation per cell of [^{3}H]leucine into acid-precipitable, emetine-insensitive radioactivity associated with the mitochondrial fraction and the relative cell volume in different chloramphenicol-pretreated and washed cultures. It should be noted that the data of relative [^{3}H]leucine incorporation per cell in Fig. 2 have been normalized, as described in Materials and Methods, for variations in efficiency of cell homogenization. This method of normalization may result in an underestimate of [^{3}H]leucine incorporation if drug treatment causes a reduction in cell size. The normal-sized control cells would in fact be expected to be easier to homogenize. It is clear from Fig. 2 that no drastic changes in the mitochondrial protein synthetic capacity per cell have occurred as a result of prolonged chloramphenicol treatment of the cultures. The observed, relatively moderate changes in the relative [^{3}H]leucine incorporation per cell appear to follow fairly closely the changes in relative cell volume. Thus, cells grown in the presence of 40 μg/ml chloramphenicol were found to be smaller than control cells, with the cell volume being 90% of control after 1–2 days of exposure, and decreasing progressively to ~60% of control after 7 days (~4.5 cell generations) of exposure. Correspondingly, the relative mitochondrial protein-labeling rate, upon removal of chloramphenicol, was 100% after 3 days (~2.7 cell generations) of drug treatment and ~60% after 7 days of treatment. For the cells grown in the presence of 200 μg/ml chloramphenicol, the relative cell volume was 78% after 3 days of exposure and 98% after 6 days (~2.2 cell generations), with the apparent relative mitochondrial protein synthesis being 111 and 104%, respectively, upon drug removal.

It should be noted that the rate of mitochondrial protein synthesis 1 h after drug removal closely parallels the cell volume both in cultures

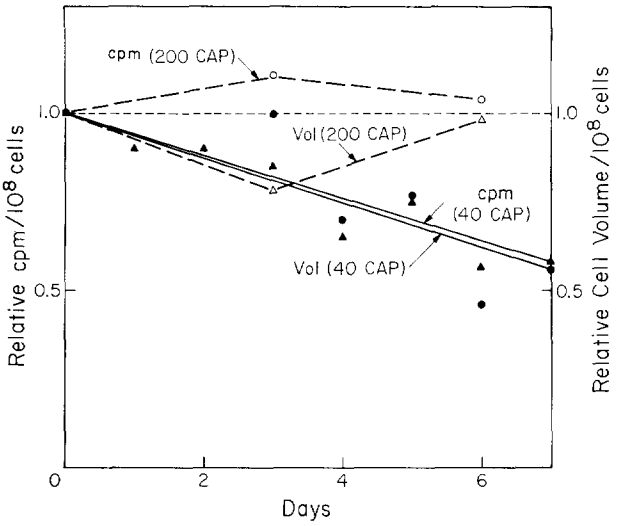

FIGURE 2 Effect of chloramphenicol (CAP) treatment of HeLa cells on cell volume and on emetine-insensitive L-[4,5-^{3}H]leucine labeling of a mitochondrial fraction. HeLa cells were grown in the absence or presence of 40 μg/ml (40 CAP) or 200 μg/ml (200 CAP) chloramphenicol for varying periods of time, washed, transferred to warm chloramphenicol-free medium, and 1 h later pulse labeled with [^{3}H]leucine for 30 min in the presence of emetine. ▲——▲, relative cell volume per 10^8 cells for cells cultured in the absence or presence of 40 μg/ml chloramphenicol (Vol [40 CAP]); ●——●, relative radioactivity incorporated per 10^8 cells for cells cultured in the absence or presence of 40 μg/ml chloramphenicol (cpm [40 CAP]); △- - -△, relative cell volume per 10^8 cells for cells cultured in the absence or presence of 200 μg/ml chloramphenicol (Vol [200 CAP]); ○- - -○, relative radioactivity incorporated per 10^8 cells for cells cultured in the absence or presence of 200 μg/ml chloramphenicol (cpm [200 CAP]).The [^{3}H]leucine incorporation data have been normalized for variations in efficiency of cell homogenization as explained in Materials and Methods. The absolute values of ^{3}H radioactivity incorporated by the non-drug-treated samples were 7500–11000 cpm/10^8 cells.

that are rapidly growing and in stationary or nearly stationary cultures. As discussed above, stationary or nearly stationary cell cultures reacquire only slowly the normal growth rate after removal of chloramphenicol.

Effects of Chloramphenicol Treatment on the Number and Size of Mitochondria and on the Amount and Arrangement of Cristae

The reversible inhibition by chloramphenicol of cell growth, the normal or near-normal rate of mitochondrial protein synthesis upon removal of chloramphenicol after prolonged drug treatment, and the previously reported normal rate of mitochondrial DNA and RNA synthesis and normal accumulation of mitochondrial DNA and malate dehydrogenase activity during the first 2.5 cell generations of growth in the presence of 40 μg/ml chloramphenicol (Storrie and Attardi, 1972) point to a high degree of mitochondrial integrity in cells grown for a number of generations in the absence of mitochondrial protein synthesis. Fig. 3 shows the morphological appearance of mitochondria in HeLa cells grown in the absence (Fig. 3 A) or presence (Fig. 3 B) of 200 μg/ml chloramphenicol for 4 days (~1.4 cell generations). The mitochondria in the two fields are virtually indistinguishable, and the organelles from both the control and the drug-treated cells appear to be equally rich in cristae mitochondriales.

The average number of mitochondrial profiles per cell cross section is not significantly affected by prolonged cell growth in the presence of 40 or 200 μg/ml chloramphenicol (Table I). The incidence of branched mitochondrial profiles observed in the sections from control cells is small (<1%) and is not significantly different in the sections from chloramphenicol-treated cells. Therefore, it is very likely that the great

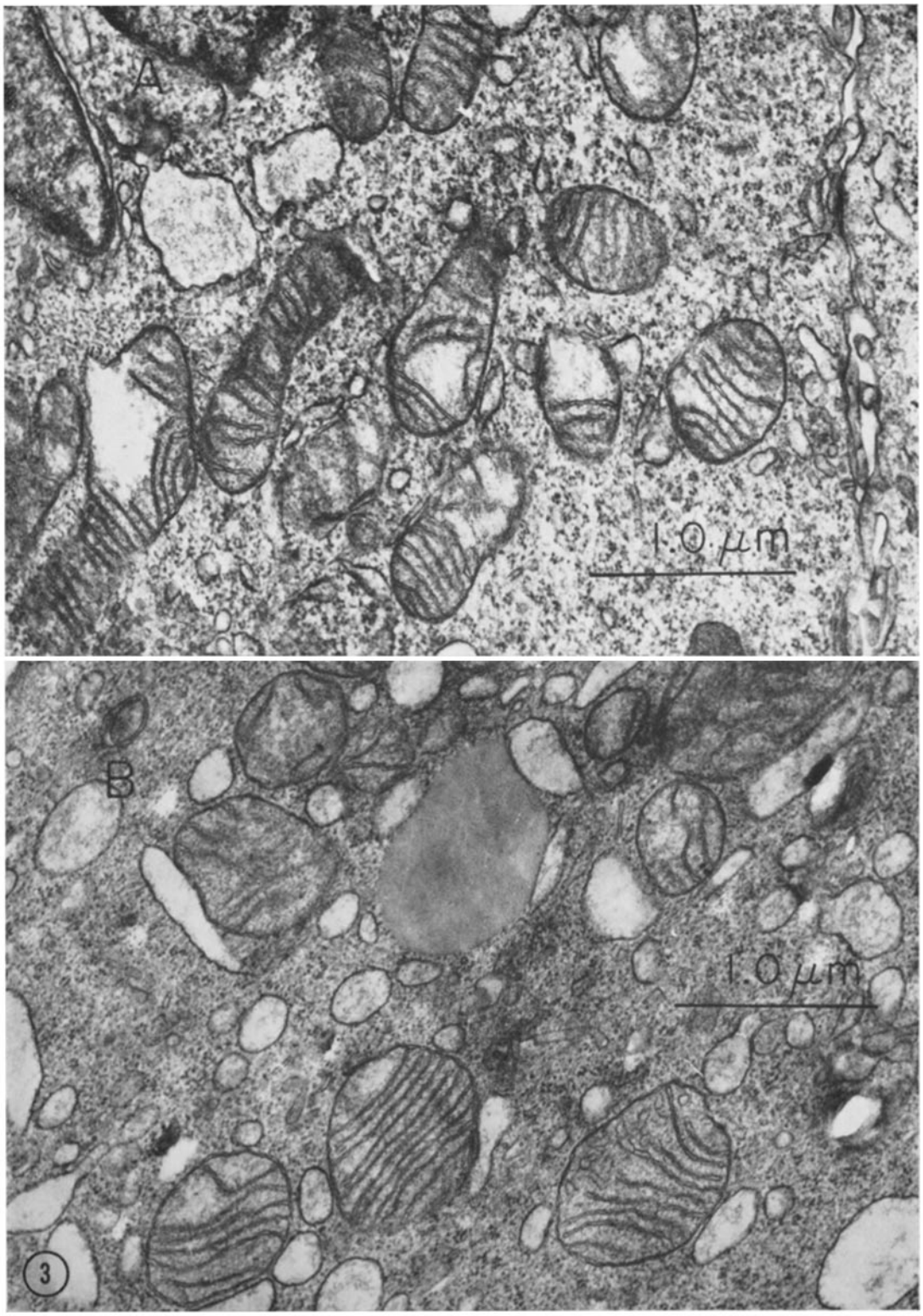

FIGURE 3 Morphological appearance of mitochondria in HeLa cells grown in the absence (A) or presence (B) of 200 μg/ml chloramphenicol. × 32,000.

majority of mitochondrial profiles scored represent individual mitochondria.

Also, the average size of the mitochondria, as estimated by the average cross-sectional area of individual mitochondrial profiles in glutaraldehyde-fixed preparations, appears to be approximately equal in control cells and in cells grown in 40 μg/ml chloramphenicol for 5 days or in 200 μg/ml chloramphenicol for 4 days (Table II). The average amount of cristae mitochondriales per mitochondrion is likewise unaffected by prolonged growth of the cells in the presence of 40 μg/ml of chloramphenicol (Table II). It should be noted that the cells grown in 200 μg/ml chloramphenicol have gone through only 1.4 generations of growth, while the cells exposed to 40 μg/ml chloramphenicol have grown for 3.8 generations in the presence of the drug.

In contrast to the normal size of mitochondria and amount of cristae, the arrangement of cristae within the mitochondrion appears to be sometimes altered by chloramphenicol inhibition of mitochondrial protein synthesis. Fig. 4 shows an area of cell exposed to 40 μg/ml chloramphenicol for 5 days, in which two of the mitochondria exhibit an unusual arrangement of cristae. The arrow in the upper half of the field points to a mitochondrial profile in which the cristae are rolled in a whorled or myelin-like configuration. In a second mitochondrion in the lower half of the field, the cristae are arranged as concentric, stirrup-shaped loops. Also found in 40 μg/ml chloramphenicol-treated cells were mitochondrial profiles in which the cristae in cross section appeared as single, free-floating circles (not shown). Similar arrangements of cristae were seen in the mitochondria of cells grown in the presence of 200 μg/ml chloramphenicol for 4 days. On the contrary, they were virtually absent in control cells. In almost 800 mitochondria from control cells scored, no mitochondrial profiles with looped or whorled cristae and only three mitochondrial profiles with cristae apparently in the form of a

TABLE I

Effect of Chloramphenicol (CAP) Treatment of HeLa Cells on the Mean Number of Mitochondrial Profiles per Cell Cross Section

Treatment	No. mitochondrial profiles/50 μm^2 cytoplasmic area	Cytoplasmic area/cell section	No. mitochondria profiles/cell section
		μm^2	
Control	17.1 ± 1.3	52.1 ± 3.0	17.8 ± 1.4
40 μg/ml CAP (5 days)	20.3 ± 1.3	48.9 ± 2.5	19.9 ± 1.3
200 μg/ml CAP (4 days)	15.7 ± 1.3	59.0 ± 4.6	18.5 ± 1.5

Number of cells scored: control, 41; 40 μg/ml CAP, 32; 200 μg/ml CAP, 40.

TABLE II

Effect of Chloramphenicol (CAP) Treatment of HeLa Cells on the Cross-Sectional Area of Mitochondrial Profiles and on the Total Length of Cristae per Unit of Mitochondrial Area or of Length of Outer Mitochondrial Membrane

Treatment	Mean cross-sectional area of mitochondrial profiles*	Total cristae length‡ per 0.25 μm^2 mitochondrial area	Total cristae length‡ per outer membrane length
	μm^2	μm	
Control	0.23 ± 0.014	3.63 ± 0.31	1.43 ± 0.13
40 μg/ml CAP (5 days)	0.25 ± 0.018	3.80 ± 0.16	1.79 ± 0.22
200 μg/ml (4 days)	0.23 ± 0.021	4.16 ± 0.32	1.69 ± 0.21

* Area data are the mean values of the ratios of total mitochondrial area to mitochondrial number per cell for 41 control cells, 32 cells treated with 40 μg/ml CAP, and 40 cells treated with 200 μg/ml CAP.

‡ Cristae measurements were made on sets of 10–20 mitochondria from five to eight cells for each group; the averages refer to the data obtained for the individual cells.

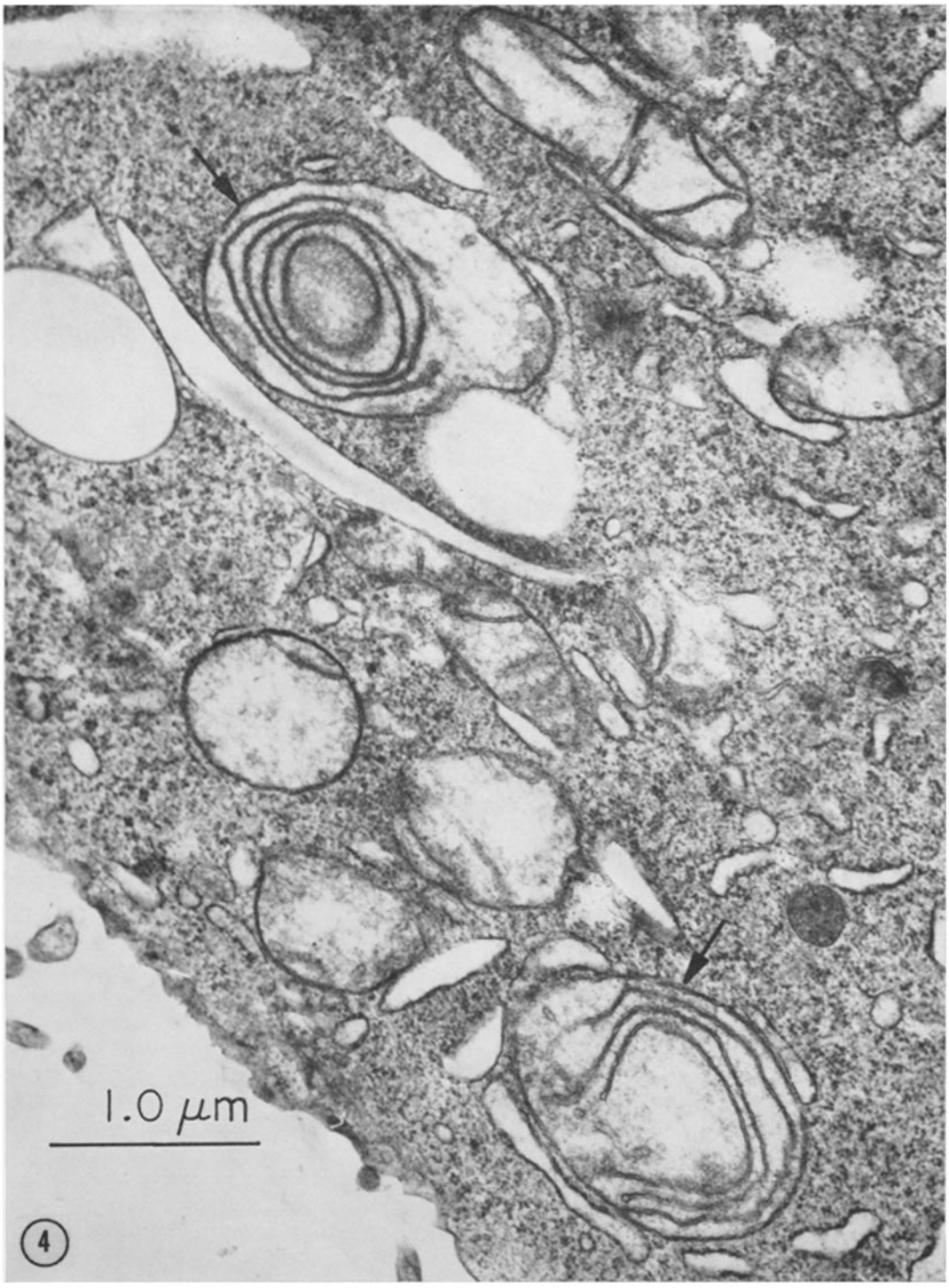

FIGURE 4 Mitochondria with whorled cristae arrangement in a HeLa cell grown in the presence of chloramphenicol (40 μg/ml). × 32,000.

TABLE III

Frequency of Mitochondrial Profiles with Altered Arrangement of Cristae in Control and Chloramphenicol (CAP)-treated HeLa Cells

Treatment	Arrangement of cristae		
	Single circular	Looped	Whorled
Control	0.4% (3)	<0.1%	<0.1%
40 μg/ml CAP (5 days)	2.1% (13)	0.8% (5)	1.1% (7)
200 μg/ml CAP (4 days)	2.3% (19)	1.1% (9)	1.2% (10)

Number of mitochondrial profiles scored: control, 798; 40 μg/ml CAP, 615; 200 μg/ml CAP, 843.

single, free-floating circular element were seen (Table III).

Mitochondrial profiles with an altered arrangement of cristae altogether constitute ~4–5% of the total mitochondrial populations in cells treated with either 40 or 200 μg/ml chloramphenicol (Table III). This number may somewhat underestimate the incidence of mitochondria with altered cristae, since some areas of altered cristae may not be seen in a given mitochondrial cross section. As a matter of fact, in occasional mitochondrial profiles the cristae are arrayed in an orthodox manner in a portion of the mitochondrion, while in the remainder the cristae are arranged in a whorled figure.

Fig. 5 shows mitochondria stained with DAB and counterstained lightly with lead citrate in cells grown in the absence (Fig. 5 A) or the presence (Fig. 5 B) of 40 μg/ml chloramphenicol for 5 days. The mitochondria from drug-treated cells contain DAB-positive circular or whorled cristae, showing that these arrangements of cristae are similar to other portions of the inner mitochondrial membrane in their staining for cytochrome oxidase. It should be noted that the DAB staining of the cristae, because of the double membrane which constitutes these projections, is more intense than that of the portions of the inner membrane which line the outer membrane. In control experiments, the DAB-staining reaction for cytochrome oxidase was found to be completely sensitive to the cytochrome *c* oxidase inhibitor, potassium cyanide.

DISCUSSION

In the present work, the effect on the formation of mitochondria in HeLa cells of selective inhibition of mitochondrial protein synthesis by chloramphenicol at concentrations of 40 or 200 μg/ml has been investigated. Either concentration of chloramphenicol inhibits overall mitochondrial protein synthesis by >90%, and, furthermore, prevents the synthesis of all electrophoretic components of the proteins synthesized in mitochondria (Lederman and Attardi, submitted for publication). The higher concentration of chloramphenicol was utilized here because of the evidence that protein synthesis required for the initiation of bacterial DNA replication (Lark and Lark, 1966) and for the synthesis of progeny replicative forms of φX174 (Levine and Sinsheimer, 1969) is inhibited only by relatively high chloramphenicol concentrations (>100 μg/ml). The general conclusion of the present investigation is that mitochondria form normally in cells grown for periods up to 4–5 days in the presence of 40 or 200 μg/ml chloramphenicol.

Effects of Chloramphenicol on Cell Growth and their Reversibility

In this work, HeLa cells maintained in modified Eagle's medium with 5% undialyzed calf serum in the presence of 40 μg/ml chloramphenicol continued to grow for several days, going through approximately four cell generations in 5 days, with the growth rate decreasing sharply thereafter. Under the present conditions, the growth capacity of HeLa cells exposed to 40 μg/ml chloramphenicol appeared to be appreciably greater than earlier reported for cells maintained in medium with dialyzed calf serum (Storrie and Attardi, 1972).

The observation made here that HeLa cells increase 4-fold in number and total mass in 4 days in the presence of 200 μg/ml chloramphenicol is contrary to a previous report by Firkin and Linnane (1968) that HeLa cells do not grow in the presence of the above concentration of the drug because of a direct inhibition of oxidative phosphorylation. However, the less than normal initial growth rate of HeLa cells exposed to 200 μg/ml chloramphenicol, in contrast to the normal initial growth rate in the presence of 40 μg/ml chloramphenicol, and the more rapid cessation of growth may indeed reflect a direct inhibition of oxidative phosphorylation.

Cell growth inhibition by chloramphenicol at 40 μg/ml is readily reversible, upon drug removal, during the first 5 days of treatment, with the recovery of the normal growth rate occurring

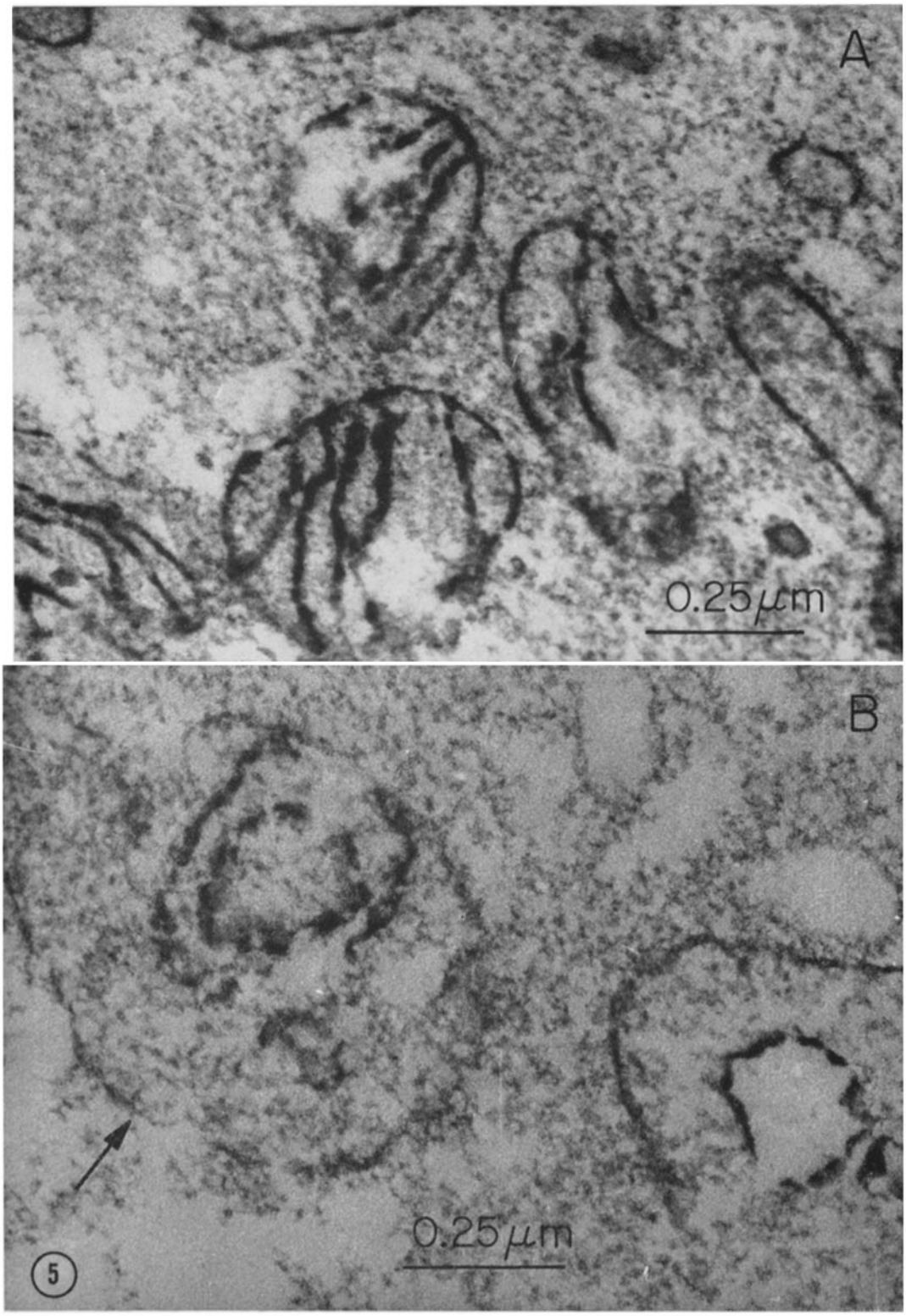

FIGURE 5 DAB-stained mitochondria in HeLa cells grown in the absence (A) or presence (B) of 40 μg/ml chloramphenicol for 5 days. Note the staining of the altered cristae in the organelles of drug-treated cells. The sections have been treated for 30 s with lead citrate. × 102,000.

immediately or in 1–2 days. Later, however, when the cultures in the presence of chloramphenicol have reached a stationary or near-stationary phase, a normal cell growth rate resumes only slowly, 4 or more days after removal of the drug. The increasing time required for the recovery of normal growth rate after drug removal presumably reflects, at least in part, the progressive reduction in cloning efficiency observed during prolonged chloramphenicol treatment of HeLa cells. It is likely that this reduction is not a result of damage to the mitochondrial protein-synthesizing apparatus, as mitochondrial protein synthesis resumes at a normal per cell rate, 1 h after drug removal, even after a time of chloramphenicol treatment when the cloning efficiency is <5%.

Effects of Choramphenicol Pretreatment on Mitochondrial Protein Synthesis

The normal rate of mitochondrial protein synthesis observed after prolonged treatment of cultures with chloramphenicol is consistent with the normal formation of 12 and 16S mitochondrial ribosomal RNAs and the normal sedimentation profile of mitochondrial RNA, previously described in drug-treated cultures (Storrie and Attardi, 1972). Presumably, the stability of mitochondrial RNA is normal in mitochondria where translation and transcription are uncoupled. The normal or near-normal rate of mitochondrial protein synthesis after drug removal suggests that the proteins involved in the assembly of the mitochondrial protein-synthesizing machinery are made extramitochondrially, regardless of the origin of the messenger RNA. The same conclusion has been reached for yeast (Davey et al., 1969) on the basis of the immediate recovery of the mitochondrial protein-synthesizing capacity after growth for five generations in the presence of high concentrations of chloramphenicol. Direct evidence indicating that all or most mitochondrial ribosomal proteins are synthesized in the cytoplasm in *Neurospora* (Küntzel, 1969; Lizardi and Luck, 1972) and HeLa cells (Brega and Baglioni, 1971) has also been reported. However, the possibility has not been rigorously excluded, in the present or in the previously reported experiments on yeast, that a large pool of some mitochondrially synthesized proteins required for mitochondrial protein synthesis has not been sufficiently diluted to become rate limiting.

Effect of Choramphenicol on Mitochondrial Growth and Formation

No change in the number of mitochondrial profiles per cell cross section, in the average size of the mitochondria, or in the amount of inner membrane per mitochondrion was found in cells grown in the presence of 40 μg/ml chloramphenicol for 5 days (approximately four generations) or 200 μg/ml chloramphenicol for 4 days (~1.5 generations). These observations imply that mitochondria grow and increase in number normally in the absence of mitochondrial protein synthesis, and extend our previous findings (Storrie and Attardi, 1972) indicating a normal synthesis of mitochondrial DNA, RNA, and malate dehydrogenase in the absence of mitochondrial protein synthesis. While this manuscript was in preparation, other authors (King et al., 1972) reported a normal number of mitochondria in HeLa cells after prolonged treatment with 20–40 μg/ml chloramphenicol. In yeast, the inability of chloramphenicol to block mitochondrial replication has been previously shown (Kellerman et al., 1969; Mahler and Perlman, 1971). A normal formation of mitochondria has been described in petite mutants of yeast, in which no mitochondrial protein synthesis occurs (see review by Linnane and Haslam, 1970), and even in mutants containing incompetent mitochondria DNA (Perlman and Mahler, 1970) or apparently lacking DNA completely (Nagley and Linnane, 1970).

The normal amount of cristae mitochondriales per mitochondrion found in mitochondria of cells treated with either 40 or 200 μg/ml chloramphenicol contradicts the report by Lenk and Penman (1971) of fewer cristae per mitochondrion in HeLa cells grown in the presence of 50 μg/ml chloramphenicol for 3 days. The conflict probably arises from the qualitative nature of the latter report. In general, but not in all cases (see, for example, Mahler and Perlman, 1972), in yeast petites the amount of inner mitochondrial membrane per mitochondrion has been reported to be greatly decreased (Linnane and Haslam, 1970). In mammalian cells with a much smaller mitochondrial genome size, the assembly of the inner mitochondrial membrane may be less dependent on mitochondrially synthesized protins.

In a small fraction (<5%) of mitochondria in cells exposed to either 40 μg/ml for 5 days or 200

μg/ml chloramphenicol for 4 days, the cristae were found to be arranged in a circular, loop-like or whorled configuration. Whorled cristae have been reported by others in HeLa cells (Lenk and Penman, 1971; King et al., 1972) and L cells (Soslau and Nass, 1971; King et al., 1972) treated with either chloramphenicol or ethidium bromide, and in mitochondria of aging blowfly flight muscles (Sacktor and Shimada, 1972). Whorled cristae may represent a rare rearrangement event in mitochondria depleted of mitochondrially synthesized proteins.

The present experiments give little information concerning the mode of mitochondrial biogenesis in mammalian cells. In *Neurospora*, the experiments of Luck (1963, 1965) indicate that mitochondria arise by growth and division of preexisting mitochondria. If, as is likely, mitochondria in mammalian cells similarly arise from replication of preexisting mitochondria (Storrie and Attardi, 1973), then the present work does suggest that in these cells the protein components involved in the actual replication of the mitochondrion, separate from mitochondrial functionality, derive completely from extramitochondrial synthesis.

The valued assistance of Mr. P. Koen with the electron microscopy, of Mr. J. Posakony and Dr. J. England with the DAB reaction, and of Mrs. B. Keeley is gratefully acknowledged.

This work was supported by a grant from the U. S. Public Health Service (GM-11726) and by a National Science Foundation predoctoral fellowship to Dr. Storrie.

Paper XIV in this series is M. Wu, N. Davidson, G. Attardi, and Y. Aloni, 1972, *J. Mol. Biol.* **71**:81.

Received for publication 3 August 1972, and in revised form 30 October 1972.

REFERENCES

AMALDI, F., and G. ATTARDI. 1968. *J. Mol. Biol.* **33**:737.

ATTARDI, B., B. CRAVIOTO, and G. ATTARDI. 1969. *J. Mol. Biol.* **44**:47.

BREGA, A., and C. BAGLIONI. 1971. *Eur. J. Biochem.* **22**:415.

DAVEY, P. J., R. YU, and A. W. LINNANE. 1969. *Biochem. Biophys. Res. Commun.* **36**:30.

FIRKIN, F. C., and A. W. LINNANE. 1968. *Biochem. Biophys. Res. Commun.* **32**:398.

KELLERMAN, G. M., D. R. BIGGS, and A. W. LINNANE. 1969. *J. Cell Biol.* **42**:378.

KING, M. E., G. C. GODMAN, and D. W. KING. 1972. *J. Cell Biol.* **53**:127.

KÜNTZEL, H. 1969. *Nature (Lond.).* **222**:142.

LARK, C., and K. G. LARK. 1966. *J. Mol. Biol.* **10**:120.

LENK, R., and S. PENMAN. 1971. *J. Cell Biol.* **49**:541.

LEVINE, A. J., and R. L. SINSHEIMER. 1969. *J. Mol. Biol.* **39**:655.

LEVINTOW, L., and J. E. DARNELL. 1960. *J. Biol. Chem.* **235**:70.

LINNANE, A. W., and J. M. HASLAM. 1970. *Curr. Top. Cell. Regul.* **2**:102.

LIZARDI, P. M., and D. J. L. LUCK. 1972. *J. Cell Biol.* **54**:56.

LUCK, D. J. L. 1963. *Proc. Natl. Acad. Sci. U. S. A.* **49**:233.

LUCK, D. J. L. 1965. *J. Cell Biol.* **24**:461.

MAHLER, H. R., and P. S. PERLMAN. 1971. *Biochemistry.* **10**:2979.

MAHLER, H. R., and P. S. PERLMAN. 1972. *Arch. Biochem. Biophys.* **148**:115.

NAGLEY, P., and A. W. LINNANE. 1970. *Biochem. Biophys. Res. Commun.* **39**:989.

OJALA, D., and G. ATTARDI. 1972. *J. Mol. Biol.* **65**:273.

PERLMAN, P. S., and H. R. MAHLER. 1970. *Bioenergetics.* **1**:113.

PERLMAN, S., and S. PENMAN. 1970. *Nature (Lond.).* **227**:133.

PICA-MATTOCCIA, L., and G. ATTARDI. 1971. *J. Mol. Biol.* **57**:615.

SACKTOR, B., and Y. SHIMADA. 1972. *J. Cell Biol.* **52**:465.

SELIGMAN, A. M., M. S. KARNOVSKY, H. L. WASSERKRUG, and J. S. HANKER. 1968. *J. Cell Biol.* **38**:1.

SOSLAU, G., and M. M. K. NASS. 1971. *J. Cell Biol.* **51**:514.

STORRIE, B., and G. ATTARDI. 1972. *J. Mol. Biol.* **71**:177.

STORRIE, B., and G. ATTARDI. 1973. *J. Cell Biol.* **56**:833.

VENABLE, J. H., and R. COGGESHALL. 1965. *J. Cell Biol.* **25**:407.