

# MITOCHONDRIAL PROTEIN SYNTHESIS IN HeLa CELLS

JONAS B. GALPER

From the Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461.  
Dr. Galper's present address is the National Heart and Lung Institute, Bethesda, Maryland 20514.

## ABSTRACT

HeLa cell mitochondrial proteins have been shown to be the products of two separate protein-synthesizing systems; one, the general cellular mechanism, sensitive to inhibition by cycloheximide, the other, a specific mitochondrial system subject to inhibition by low concentrations of chloramphenicol (Galper, J. B., and J. E. Darnell. 1971. *J. Mol. Biol.* 57:363). Preliminary data have suggested that a mitochondrial *N*-formyl-methionyl-tRNA (f-Met-tRNA) might be the initiator tRNA in the latter (Galper, J. B., and J. E. Darnell. 1969. *Biochem. Biophys. Res. Commun.* 34:205; 1971. *J. Mol. Biol.* 57:363).

It is demonstrated here that the synthesis of these endogenous mitochondrial proteins is also subject to inhibition by ethidium bromide and decays with a half-life of 1½–2 h in cultures incubated with low concentrations of this dye. The role of formylated f-Met-tRNA as the initiator tRNA in the synthesis of mitochondrial proteins is supported by data from several experiments. The rates of ethidium bromide inhibition of both the charging of f-Met-tRNA and of the synthesis of mitochondrial proteins are strikingly similar. Inhibition by aminopterin of the formylation of f-Met-tRNA greatly depresses the rate of mitochondrial-specific protein synthesis. In the absence of the synthesis of these proteins, respiration, the levels of cytochromes *a*-*a*<sub>3</sub> and *b*, and the number of mitochondrial cristae are decreased. The implications of these findings as they relate to mitochondrial biogenesis are discussed.

## INTRODUCTION

Considerable evidence has accumulated supporting the existence in mitochondria of the necessary components of a system for the transfer of genetic information and the synthesis of proteins. Mitochondria of fungi and animal cells have been shown to contain characteristic supercoiled DNA (Hudson and Vinograd, 1967; Guerinéau et al., 1968), ribosomal RNA's, ribosomes (Kuntzel and Noll, 1967; Vesco and Penman, 1969; Rifkin et al., 1969; Swanson and Dawid, 1970), and specific tRNA's (Barnett and Brown, 1967; Buck and Nass, 1969) including a formylated

formyl-methionyl-tRNA (f-Met-tRNA) (Galper and Darnell, 1969) shown to take part in a ribosome-mediated reaction (Galper and Darnell, 1971). Experiments with isolated mitochondria, although subject to the question of bacterial contamination, have demonstrated that incorporation of amino acids into mitochondrial protein (Roodyn, 1965; Lamb et al., 1968) was sensitive to inhibition by chloramphenicol, a drug which at low concentrations has little effect on general protein synthesis in eukaryotic cells (Linnane, 1968). Conversely, cycloheximide, a

potent inhibitor of protein synthesis in animal cells (Loeb and Hubby, 1968), had no effect on amino acid incorporation into mitochondrial preparations.

Evidence has been presented (Galper and Darnell, 1971) suggesting that in HeLa cells, over 85% of those proteins made in the presence of cycloheximide are found in the mitochondrial fraction of the cell. It was further demonstrated (Galper and Darnell, 1971) that these proteins comprise 20% of total mitochondrial proteins and range from 30,000 to 40,000 in molecular weight. Their synthesis, while unaffected by increasingly high levels of cycloheximide, was subject to inhibition by low levels of chloramphenicol.

Yeasts grown in the presence of chloramphenicol, acridine dyes, or ethidium bromide (the petite mutation) have been shown to be incapable of respiration. The mitochondria of these cells contain fewer cristae and markedly decreased levels of cytochromes *a*, *b*, *c*<sub>1</sub>, and the mitochondrial dehydrogenases (Linnane et al., 1968). Zylber, Vesco, and Penman (1969) have demonstrated that the synthesis of certain species of HeLa cell mitochondrial RNA's was sensitive to inhibition by ethidium bromide.

In this communication, experiments are presented which further elucidate the properties of a unique system for the synthesis of mitochondrial proteins. The interaction of this system with ethidium bromide, the function of formylated mitochondrial f-Met-tRNA in the initiation of the synthesis of mitochondrial proteins, and the role of these proteins in mitochondrial biogenesis are studied.

#### MATERIALS AND METHODS

HeLa cells, strain S<sub>3</sub>, were grown at 37°C in suspension culture maintained at a concentration of 2.0–6.0 × 10<sup>5</sup> cells/ml in Eagle's medium (Eagle, 1959).

##### *Labeling of Cell Proteins*

When cells were to be labeled with one or more essential amino acids, they were rinsed for 5 min in medium containing 5% dialyzed serum and lacking the appropriate amino acids, harvested, resuspended at 4.0–5.5 × 10<sup>6</sup> cells/ml in an aliquot of the same medium, and the appropriate labeled amino acid(s) added. For uniform labeling of cellular proteins, cells at 4.0–5.0 × 10<sup>5</sup> cells/ml diluted with an equal volume of leucine-free medium were incubated overnight with 0.18 μCi L-[5-6 <sup>3</sup>H]leucine (5 Ci/mmol)/liter.

Eagle's medium in powdered form (Joklik modified), methionine-free medium, horse serum, and dialyzed horse serum were obtained from Grand Island Biological Co., Grand Island, N. Y. Amino acid-free medium was prepared by the combination of solutions of carbohydrate, salts, vitamins, antibiotics, pH indicator, and the appropriate essential amino acids.

Radioactive amino acids with the following specific activities were purchased from New England Nuclear, Boston, Mass.: L-[<sup>14</sup>C]methionine (uniformly labeled) 200 μCi/μmol; L-[<sup>14</sup>C] amino acid (uniformly labeled) mixture which contained per millicurie of mixture; 80 μCi L-alanine, 70 μCi L-arginine, 80 μCi L-aspartic acid, 125 μCi L-glutamic acid, 80 μCi L-isoleucine, 140 μCi L-leucine, 60 μCi L-lysine, 80 μCi L-phenylalanine, 50 μCi L-proline, 40 μCi L-serine, 50 μCi L-threonine, 40 μCi L-tyrosine, 80 μCi L-valine. Specific activities ranged from 40 to 376 μCi/μmol.

*N*-formyl-L-[<sup>14</sup>C]-methyl methionine, 10 μCi/μmol, and L-[5-6 <sup>3</sup>H] leucine 5 Ci/μmol, were also purchased from New England Nuclear; puromycin from Nutritional International Corp., Madison, Tenn.; chloramphenicol and aminopterin from Sigma Chemical Co., St. Louis, Mo.; acrylamide, bis-acrylamide, *N,N,N',N'*-tetramethylenedionine, ethyl acetate, dicyclohexyldicarbodiimide mercaptoethanol and ninhydrin from Eastman Organic Chemical Div., Eastman Kodak Co., Rochester, N.Y.; and ammonium persulfate from the J. T. Baker Chemical Co., Phillipsburg, N. J.

##### *Harvesting and Fractionation of Cells*

Cells were collected by centrifugation at 4°C (at 400 *g*), washed twice in cold Earle's saline (Earle, 1943), resuspended in SET buffer (0.25 M Tris, pH 7.4, 0.25 M sucrose, and 0.01 M EDTA) at a concentration of 3.0–5.0 × 10<sup>7</sup> cells/ml, and disrupted by 10–15 strokes of a stainless steel Dounce homogenizer (tolerance of +0.001 inches) which, although sufficient to disrupt less than 50% of the cells, left mitochondria and nuclei relatively intact.

Nuclei and whole cells were removed by centrifugation at 800 *g* for 2 min, the pellet was washed in SET buffer, and the supernate and washings were pooled to give the whole cytoplasmic extract which in turn was separated into the postmitochondrial supernate and the mitochondrial pellet by centrifugation at 8,000 *g* for 10 min at 4°C. The mitochondrial pellet was washed and layered over a 15–30% sucrose gradient overlying 5 ml of 55% sucrose in TM buffer (0.01 M Tris, 0.00015 M MgCl<sub>2</sub>). Centrifugation was carried out for 60–90 min at 60,000 *g* using either the SW 25.1 or SW 27 rotor (Beckman Instruments, Inc., Palo Alto, Calif.).

The banded mitochondria were collected, and the

suspension was made 0.25 M in sucrose and pelleted by centrifugation for 10 min at 8,000 g.

### *Preparation and Electrophoresis of Mitochondrial Proteins*

Whole mitochondria were taken up in a solution of 1% sodium dodecyl sulfate (SDS)-1% mercaptoethanol and heated at 95°C for 1–1.5 min to ensure solubilization of proteins. Samples were dialyzed at least 2 h against 1–2 liters of sample buffer (15% sucrose, 0.1% SDS, 1% mercaptoethanol, 0.005 M Na phosphate pH 7.0) and protein concentration was determined by the method of Lowry et al. (1951).

Polyacrylamide gels were prepared using the phosphate-SDS system (Maizel, 1969). Pretreatment of gels with a sulfhydryl agent was carried out by the method of Strauss et al., 1969.

Samples containing 150–200  $\mu\text{g}$  protein were subjected to electrophoresis on 7.5% acrylamide gels at 5–8 mA/gel at room temperature. The gel was crushed in a semiautomatic fractionator (Maizel, 1966), 10 ml of Bray's scintillation fluid (Bray, 1960) were added to each fraction, and samples counted for 4 min in an Ansitron liquid scintillation counter.

In order to make quantitative comparisons between the patterns of radioactive labeling of proteins in two separate gels, experiments were performed on aliquots of the same culture labeled to equilibrium by overnight growth in [ $^3\text{H}$ ]leucine. All gels were normalized to equal concentrations of protein by total recovery of  $^3\text{H}$ .

### *Methods for Studying Cytochromes and Respiration*

Cells were grown for two generations in the presence of either 15–20  $\mu\text{g}/\text{ml}$  chloramphenicol, or 0.25–0.15  $\mu\text{g}/\text{ml}$  ethidium bromide, rinsed, and resuspended at  $1 \times 10^7$  cells/ml in TS buffer (0.01 M Tris, 0.25 M sucrose). The sample was divided and a base-line difference spectrum was determined (620–400 m $\mu$ ) with a dual beam spectrophotometer (Phoenix Precision Instrument Div. Virtis Co., Inc., Gardiner, N. Y.). One aliquot was then reduced with a few crystals of dithionite, the difference spectrum was determined, and baseline subtracted.

For determination of the respiratory rate of whole cells, cells were collected and added to a small volume of incubation medium (0.05–0.02 ml; 250 mM sucrose, 5 mM KCl, 10 mM Tris phosphate, 5 mM Tris HCl, pH 7.4) in the cell of a micro-oxygen electrode system (total volume 0.1 ml), designed and built by Dr. Cyril L. Moore, Albert Einstein College of Medicine. The rate of oxygen consumption was determined, taking the initial  $\text{O}_2$  concentration of a saturated solution as 240  $\mu\text{mol}/\text{ml}$ , and data was

plotted as  $\mu\text{mol O}_2$  consumed/min/mg protein/0.1 ml. Corrections were made for background of the system.

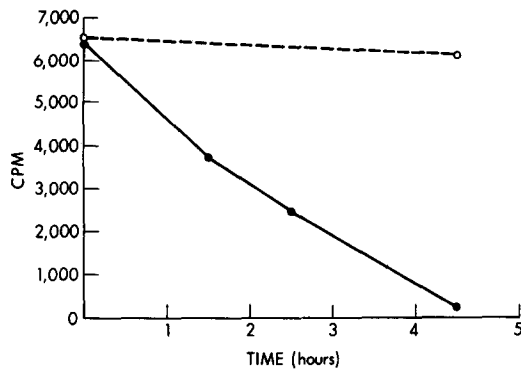
## RESULTS

To study the effects of ethidium bromide on the cycloheximide-insensitive mitochondrial protein-synthesizing system, aliquots of a culture were taken at various intervals after the addition of the dye (1  $\mu\text{g}/\text{ml}$ ), incubated in the presence of cycloheximide (200  $\mu\text{g}/\text{ml}$ ) and labeled with a mixture of [ $^{14}\text{C}$ ]amino acids. Total  $^{14}\text{C}$  counts incorporated into mitochondrial proteins prepared from each aliquot (residual cycloheximide-resistant protein synthesis) were plotted as a function of the time of incubation in ethidium bromide. Under these conditions, the capacity of the cell to synthesize cycloheximide-resistant mitochondrial proteins decayed with a half-life of less than 2 h (Fig. 1). To study the effect of ethidium bromide on general cellular protein synthesis, aliquots of the same culture were withdrawn at given times and the capacity of cells to incorporate [ $^{14}\text{C}$ ]amino acids into postmitochondrial supernatant proteins was determined. As seen in Fig. 1, ethidium bromide treatment alone had no effect on the synthesis of cellular proteins for up to 4½ h. Hence, as with chloramphenicol, short exposure of cells to ethidium bromide appears specifically to affect only the mitochondrial protein-synthesizing system. Unlike inhibition by chloramphenicol, inhibition by ethidium bromide was time dependent. Analysis on polyacrylamide gels of mitochondrial proteins revealed that ethidium bromide was indeed inhibiting the synthesis of those mitochondrial proteins whose synthesis is insensitive to cycloheximide. After exposure of cells for 4 h to the dye, this inhibition was complete (Fig. 2).

At the concentrations of dye used in these experiments (1  $\mu\text{g}/\text{ml}$ ) cells were capable of growth through one cell division over a 36-h period as determined by cell count and increase in total cellular protein. After two such cycles, cells ceased to divide and rapidly degenerated. Control cultures exhibited a doubling-time of 24 h.

### *Function of F-Met-tRNA in the Mitochondrion*

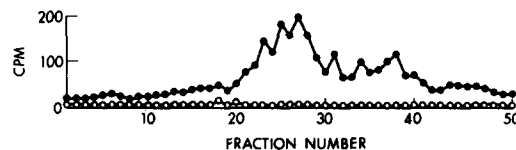
It was shown (Galper and Darnell, 1969) that HeLa cell mitochondria contain formylated f-Met-tRNA. To determine whether or not the



**FIGURE 1** Effect of ethidium bromide on cycloheximide-resistant mitochondrial protein synthesis.  $1.2 \times 10^9$  cells were labeled overnight with 1 mCi [ $^3\text{H}$ ]leucine. Ethidium bromide ( $1 \mu\text{g}/\text{ml}$ ) was added to the culture, and four aliquots of  $2 \times 10^8$  cells were each taken at the times indicated and treated for 5 min with cycloheximide ( $200 \mu\text{g}/\text{ml}$ ). Rinsing and labeling of cells ( $1.6 \mu\text{Ci}/\text{ml}$  [ $^{14}\text{C}$ ]amino acid mixture for 25 min) and preparation of mitochondria from each aliquot were done as described under **MATERIALS AND METHODS**. Two further aliquots were taken, one at zero time and one at  $4\frac{1}{2}$  h, rinsed, labeled with [ $^{14}\text{C}$ ]amino acids ( $1.6 \mu\text{Ci}/\text{ml}$  for 25 min) in the absence of cycloheximide, and the postmitochondrial supernate was prepared (see **Materials and Methods**). Mitochondria were solubilized in 1% SDS-1% mercaptoethanol at  $95^\circ\text{C}$  for 1 min and analyzed on 7 $\frac{1}{2}$ % polyacrylamide gels at 8 mA/gel. Gel fractionation and radioactive counting of samples were carried out as described in **Materials and Methods**. Time points are taken from the moment of addition of ethidium bromide until the middle of the 25-min labeling period. Ethidium bromide and cycloheximide were present in all appropriate rinsing and labeling media. All samples were corrected for equal concentrations of protein by the relative recovery of [ $^3\text{H}$ ]. ●—●, labeled mitochondrial proteins in the presence of ethidium bromide plus cycloheximide; ○—○ labeled postmitochondrial supernatant proteins, ethidium bromide alone.

turnover of the f-Met moiety was indeed a ribosome-mediated reaction, the effect of puromycin on the charging of formylated f-Met-tRNA was studied. Bretscher and Marcker (1966) and Leder and Bursztyl (1966) have demonstrated that in systems in which formyl-methionine is the initiator of protein synthesis, the addition of puromycin leads to the formation of *N*-formyl-methionyl-puromycin (f-Met-puromycin).

Ethyl acetate extracts of HeLa cell cultures labeled with [ $^{14}\text{C}$ ]methionine and treated with puromycin revealed a peak of radioactivity with an electrophoretic mobility identical to that



**FIGURE 2** Inhibition of cycloheximide resistant mitochondrial protein synthesis in the presence of ethidium bromide.  $5.0 \times 10^8$  cells were labeled overnight with  $300 \mu\text{Ci}$  of [ $^3\text{H}$ ]leucine. Ethidium bromide ( $1 \mu\text{g}/\text{ml}$ ) was added to one-half of the culture and the other half was maintained as a control. Both cultures were further incubated for 4 h and at that time treated with cycloheximide ( $200 \mu\text{g}/\text{ml}$ ) for 5 min. Subsequent pulse labeling of cells with a mixture of [ $^{14}\text{C}$ ]amino acids ( $1 \mu\text{Ci}/\text{ml}$  for 25 min), preparation of mitochondria, solubilization of mitochondrial proteins, and gel electrophoresis were carried out as described in **Materials and Methods**. ●—●, labeled mitochondrial proteins, cycloheximide alone; ○—○, labeled mitochondrial proteins, cycloheximide plus ethidium bromide.

of synthetically prepared (structure determined by mass spectroscopy) f-Met-puromycin (Galper and Darnell, 1971).

Mild acid treatment of f-Met-puromycin should lead to preferential hydrolysis of the formamide linkage and release of methionyl-puromycin (Met-puromycin). Radioactivity was eluted from the presumed f-Met-puromycin region of chromatograms, subjected to mild acid, and rechromatographed (upper panel, Fig. 3) in parallel with a sample of standard f-Met-puromycin which had been similarly treated (middle panel, Fig. 3). The hydrolysate of both extracts exhibited identical electrophoretic mobilities.

Further treatment of presumed Met-puromycin with strong acid at elevated temperatures was shown to cleave the peptide bond, releasing free methionine (Galper, 1970). Hence, standard f-Met-puromycin bears physical and chemical properties identical to those of ethyl acetate-extractable material from puromycin-treated HeLa cell cultures.

In order more clearly to establish the role of f-Met-tRNA in mitochondrial protein synthesis, experiments were designed to demonstrate whether or not inhibitors of mitochondrial protein synthesis, e.g. ethidium bromide, affected the rate of charging of f-Met-tRNA and whether or not inhibitors of the formylation of f-Met, e.g. aminopterin, affected the synthesis of cycloheximide-resistant mitochondrial proteins. For these studies, the rate of mitochondrial protein

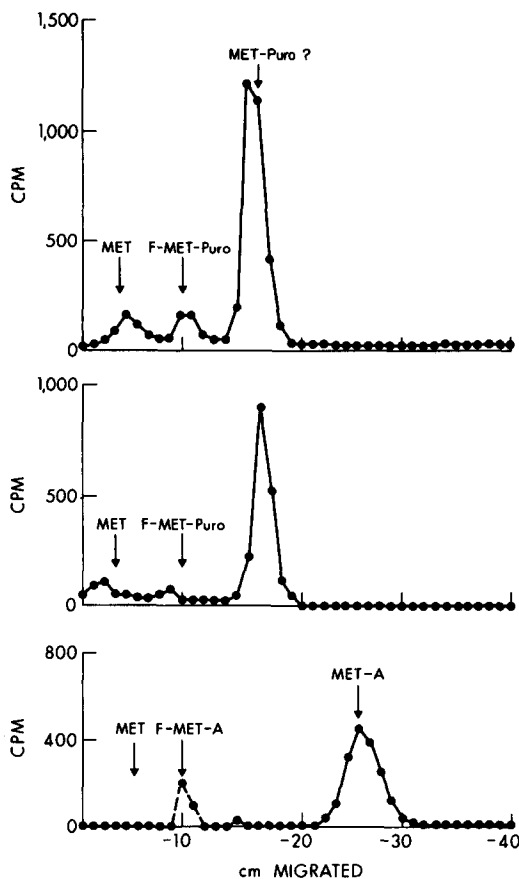


FIGURE 3 Product of mild acid hydrolysis of presumed f-Met-puromycin.  $1.8 \times 10^8$  cells were labeled with [ $^{14}\text{C}$ ]methionine (0.01  $\mu\text{Ci}/\text{ml}$ ) for 2 min, treated with puromycin (0.15 mg/ml) for 14 min, homogenized, and acidified to pH 5.5. The homogenate was extracted with ethyl acetate, and the extract was subjected to high voltage electrophoresis in parallel with a sample of standard [ $^{14}\text{C}$ ]f-Met-puromycin. Material from the presumed f-Met-puromycin peak was eluted with methanol, the solvent distilled, made 0.2 N in HCl (Sheehan and Yang, 1958), sealed under a vacuum in glass ampules, and incubated at 37°C for 36 h. Samples were then neutralized and subjected to electrophoresis (70 min, 3,000 V) in pyridine-acetate buffer pH 3.5; chromatograms were cut into strips, eluted with  $\text{NH}_4\text{OH}$ , and radioactivity was determined in Bray's scintillant (Bray, 1960). Arrows within figures indicate mobilities of standard compounds. (Upper panel) HeLa cell extracts subjected to mild acid hydrolysis. (Middle panel) Standard [ $^{14}\text{C}$ ]f-Met-puromycin eluted and subjected to similar treatment. (Lower panel) Standards (Formyl-methionyl-adenosine) chromatographed in parallel and prepared as previously described (Galper and Darnell, 1969). These standards migrated as expected from structural and electro-

TABLE I  
Effect of Preincubation of Cells with Ethidium Bromide on f-Met-puromycin Production: Comparison with decay in mitochondrial protein synthesis

Preincubation period	f-Met-puromycin	Control	Residual protein synthesis
min	cpm	%	%
0	17,500	100*	100
55	13,350	76	75
85	10,860	64	62
145	6,475	37	41
265	1,300	7	5

$10.0 \times 10^8$  cells were made 1  $\mu\text{g}/\text{ml}$  in ethidium bromide. At the times indicated, aliquots of  $2 \times 10^8$  cells were removed, rinsed, labeled for 2 min with [ $^{14}\text{C}$ ]methionine (0.2  $\mu\text{Ci}/\text{ml}$ ), and half the culture was treated for 14 min with 0.25 mg/ml puromycin. The cells were then poured onto frozen Earle's saline solution (Earle, 1943), and after homogenization and acidification to pH 5.5, the ethyl acetate extract was subjected to paper electrophoresis (pH 3.7, 3000 V, 2 h) as described (Fig. 3). The total counts migrating as f-Met-puromycin in each extract were determined. As in Fig. 1, each time point was taken as the time elapsed from the addition of ethidium bromide until the middle of the 14-min labeling period.

\* From data presented in Fig. 2.

synthesis in the presence of ethidium bromide could be measured directly as in Fig. 1. As a measure of f-Met-tRNA function, the relative rate of f-Met-puromycin formation was an accurate and easily accessible parameter since, under control conditions, the rate of f-Met-puromycin production was found to be linear with time for at least 14 min (Galper, 1970).

In an experiment similar to that shown in Fig. 1, aliquots of a culture were removed at specific periods after the addition of ethidium bromide. Each aliquot was first labeled with methionine, then incubated with puromycin for 14 min, and the total synthesis of f-Met-puromycin was determined. As is seen from the data in Table I, not only does ethidium bromide inhibit f-Met-puromycin formation, but in the presence of ethidium bromide, both mitochondrial-specific protein synthesis and f-Met-puromycin formation decay at rates which are strikingly similar.

chemical similarities with f-Met-puromycin, and Met-puromycin, respectively.

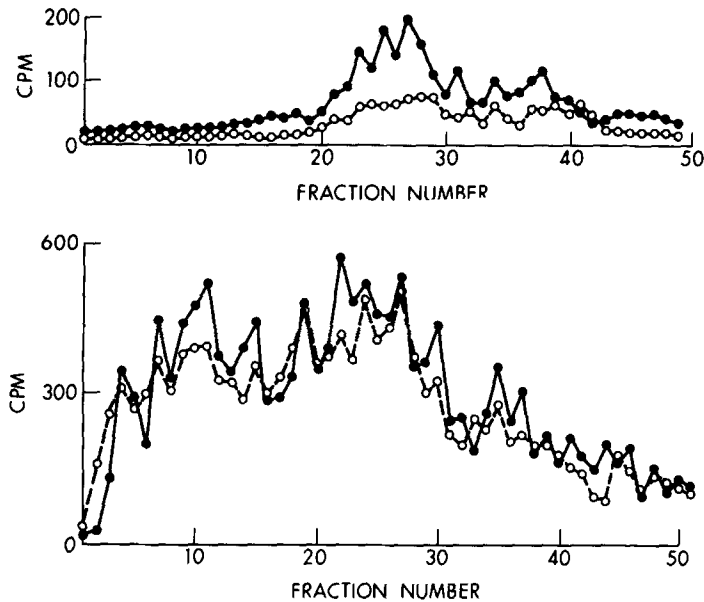


FIGURE 4 (Upper Panel) Effect of aminopterin on the synthesis of cycloheximide-resistant mitochondrial proteins. One-half of a culture,  $4.2 \times 10^8$  cells, which had been labeled overnight with  $330 \mu\text{Ci}$  [ $^3\text{H}$ ]-leucine, was made  $10^{-4}$  M in aminopterin and maintained at  $37^\circ\text{C}$  for 20 min. Both cultures were subsequently treated with cycloheximide and labeled for 25 min with [ $^{14}\text{C}$ ]amino acids ( $1.8 \mu\text{Ci}/\text{ml}$ ), and mitochondrial proteins were prepared as described previously. ●—●, mitochondrial proteins, cycloheximide alone; ○—○, mitochondrial proteins, aminopterin plus cycloheximide. (Lower Panel) Effect of aminopterin on general cellular protein synthesis.  $4.2 \times 10^8$  cells labeled overnight with  $330 \mu\text{Ci}$  [ $^3\text{H}$ ]leucine were taken from the same culture and treated similarly, except that cycloheximide was not added. Postmitochondrial supernate was prepared from cells of both cultures as described previously. An aliquot of each supernate was heated to  $95^\circ\text{C}$  (1.5 min) in the presence of 1% SDS-1% mercaptoethanol, and samples were prepared for electrophoresis in the same manner as for mitochondrial proteins. ●—● Proteins, postmitochondrial supernate of control culture. ○—○ Proteins, postmitochondrial supernate of aminopterin-treated culture.

The effect of inhibition of the formylation of f-Met-tRNA on mitochondrial protein synthesis was studied in cells incubated for 25 min in the presence of aminopterin (conditions under which 86% of f-Met formylation is inhibited; Galper, 1970; Galper and Darnell, 1969). A significant decrease in the level of cycloheximide-insensitive mitochondrial protein synthesis could be demonstrated (Fig. 4, upper panel). However, it was not possible to demonstrate complete inhibition. As will be discussed, incomplete inhibition might be explained by the ability of unformylated Met-tRNA to initiate synthesis at a decreased rate.

General cellular protein synthesis, however, was unaffected by incubation of cells for 25 min with aminopterin (Fig. 4, lower panel). Hence, for short incubation periods the effects of aminopterin appear to be specific for mitochondrial

protein synthesis in the same way that chloramphenicol and ethidium bromide are.

#### *Effect of Chloramphenicol and Ethidium Bromide on the Structure and Function of Mitochondria*

Reports from several laboratories have demonstrated that periods of growth in the presence of ethidium bromide and/or chloramphenicol led to a fall in the levels of cytochromes *a-a<sub>3</sub>* and *b*, while the levels of cytochrome *c* rose (King *et al.*, 1972; Soslau and Nass, 1971; Firkin and Linnane, 1968). The mitochondrial cristae in these cells were found to be significantly decreased in number (Lenk and Penman, 1971). It has been shown in our studies that in the absence of detectable mitochondrial protein synthesis, cell

division is able to proceed through two further cell cycles at a decreased rate (see above) (Galper and Darnell, 1971). These cells were found to respire at only 28% of the level of control cells (Fig. 5).

Fig. 6 reveals that after two generations of growth in the presence of either chloramphenicol or ethidium bromide, levels of cytochrome *c* (418 nm) remained unchanged while an almost complete disappearance of cytochrome *a-a<sub>3</sub>* (446 nm), and a decrease in cytochrome *b* (425 nm) were observed. The presence of cytochrome *b<sub>5</sub>* in HeLa cell endoplasmic reticulum probably accounts for the small amounts of this cytochrome seen in these studies.

Similarly, electron micrographs of mitochondria isolated from cells grown in the presence of these inhibitors for two generations (Galper, 1970, Lenk and Penman, 1971), reveal that mitochondria appear to be markedly altered in the quality and quantity of their cristae. In most cases, cristae are either absent (decreased in number), or thin and narrowed.

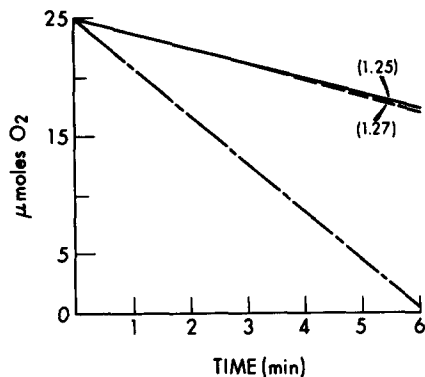


FIGURE 5 Rate of respiration of HeLa cells treated with chloramphenicol or ethidium bromide. A culture of  $7.5 \times 10^7$  cells was divided into three aliquots. Each aliquot was diluted to  $1.5 \times 10^5$  cells/ml, and incubated either through two generations (48 h) as a control without additions, or for 72 h in the presence of  $15 \mu\text{g/ml}$  ethidium. After incubation,  $3 \times 10^7$  cells from each culture were harvested without washing and the whole pellet was added directly to the chamber of an oxygen electrode as described in Materials and Methods. After determination of the rate of oxygen utilization, the sample was removed and protein concentration determined on an aliquot by the method of Lowry et al. (1951). The rate of respiration was plotted as  $\mu\text{mol O}_2$  consumed/min/mg protein/0.1 ml sample. —, control cells, rate 4.46; — — —, ethidium bromide treated cells, rate 1.25; - - -, chloramphenicol treated cells, rate 1.27.

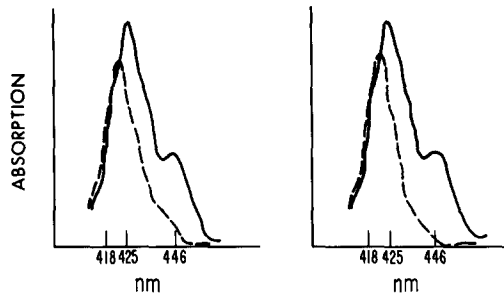


FIGURE 6 Spectral analysis of cytochromes in HeLa Cells grown in the presence of chloramphenicol or ethidium bromide. After incubation for two generations, (72 h),  $3 \times 10^7$  cells from each culture were washed and resuspended in 2 ml TM buffer, and 1 ml of cells was added directly to each of two quartz cuvettes. Difference spectra were determined as noted in MATERIALS AND METHODS. Spectra were corrected to equal concentrations of protein and plotted relative to the spectrum of the control culture. — control cells. - - - drug-inhibited cells. (Left Panel)  $15 \mu\text{g/ml}$  chloramphenicol. (Right Panel)  $0.15 \mu\text{g/ml}$  ethidium bromide.

Thus, in the absence of mitochondrial protein synthesis, mitochondria, while present, do not have the capacity to carry on normal respiration. While outer membranes remain intact, these mitochondria appear to be unable to assemble active inner membranes.

## DISCUSSION

Previously reported studies (Galper and Darnell, 1971) suggest that 20% of animal cell mitochondrial proteins are the product of a specific and unique mitochondrial protein-synthesizing system. Unlike that of other cellular proteins, their synthesis: (a) is specifically subject to inhibition by low concentrations of chloramphenicol and ethidium bromide; (b) proceeds at a reduced rate in the presence of aminopterin under conditions where the synthesis of cellular proteins is unaffected; and (c) appears to be initiated by a formylated mitochondrial f-Met-tRNA.

Zylber, Vesco, and Penman (1969) and Vesco and Penman (1969) have demonstrated the presence of a class of HeLa cell mitochondrial RNA's, 12S and 21S, whose synthesis is subject to inhibition by low concentrations of ethidium bromide. In the presence of the dye, the synthesis of these RNA's decays rapidly within 30 min while total 21S and 12S RNA decays with a time course

only slightly less than that for mitochondrial protein synthesis shown in Fig. 1. Hence protein synthesis is able to continue at least for a short time in the absence of new synthesis of these RNA's. Perlman and Penman (1970), and Montencourt and Dubin (1970) have demonstrated that these 12S and 21S mitochondrial RNA's may be associated with animal-cell mitochondrial ribosomes.

Several laboratories have demonstrated that HeLa cell mitochondria may contain not only ribosomes, but also polysomes and an mRNA with properties quite similar to those described above. Perlman and Penman (1970) have demonstrated mitochondrial ribonucleoprotein particles which, when treated with RNase, yielded a 55S particle containing both 12S and 21S RNA's. Both chloramphenicol and ethidium bromide inhibited the appearance of these particles. The data suggests that these particles are mitochondrial polysomes and that pulse-labeled proteins associated with them represent nascent mitochondrial proteins. Lederman and Attardi (1970) have prepared a mitochondrial fraction from HeLa cells which will support chloramphenicol-sensitive protein synthesis. This activity is not present in cells pretreated with ethidium bromide. Most recently, evidence (Perlman *et al.*, 1973) suggesting the presence of a mitochondrial messenger RNA has been presented.

In such a system ethidium bromide might act directly on the mitochondrial DNA (Mt-DNA). Intercalation between the bases of supercoiled Mt-DNA (Bauer and Vinograd, 1968) could render it incapable of transcription. This would account for inhibition of protein synthesis, inhibition of synthesis of 12S and 21S RNA, and the inability of the mitochondrial system to form an initiation complex with the subsequent turnoff of the f-Met-puromycin reaction. However, the data is also consistent with the interference of ethidium bromide with energy-utilization in the mitochondrion. Interaction of its polycyclic hydrocarbon structure with the outer membrane of the mitochondrion might interfere with energy transport and production, and result in turnoff of energy-dependent intramitochondrial processes.

The specific effect of chloramphenicol is probably best understood if one postulates that the mitochondrial ribosome bears more structural resemblance to that of prokaryotic cells than to cytoplasmic ribosomes. This is not unreasonable in

a system initiated by a formylated f-Met-tRNA. f-Met-tRNA is implicated as the initiator tRNA of mitochondrial protein synthesis by several experiments: (a) probable common site of action: formylated f-Met-tRNA is found exclusively in the mitochondrion; mitochondrial-specific protein synthesis is also likely to be intramitochondrial; (b) formylated f-Met-tRNA is not required for general cellular protein synthesis (Galper and Darnell, 1970), yet ribosome-mediated f-Met turnover can be demonstrated in the cell; (c) the kinetics of inhibition of f-Met turnover and mitochondrial protein synthesis are strikingly similar in the presence of ethidium bromide; (d) inhibitors of the formylation of f-Met-tRNA, e.g. aminopterin, retard mitochondrial protein synthesis.

Bretscher and Marcker (1966) have shown that the *in vitro* f-Met-puromycin reaction as well as peptide bond formation are slowed in the presence of unformylated f-Met-tRNA. This is not in agreement with the work of Eisenstadt and Lengyel (1966), however, who demonstrated complete inhibition of *in vivo* bacterial protein synthesis in the presence of trimethoprim. Work by Smith and Marcker (1970), Brown and Smith (1970), Housman *et al.* (1970), and others has demonstrated that a Met-tRNA which can be formylated by bacterial extracts is involved in the initiation of animal cell protein synthesis. Hence, unformylated mammalian mitochondrial f-Met-tRNA might be capable of initiation of synthesis at a reduced rate because of the less favorable stereochemical and electrophilic character of the unformylated methionyl residue.

Data have been presented suggesting that normal mitochondrial biogenesis requires the interaction of the products of both cytoplasmic and mitochondrial systems. In the absence of mitochondrial protein synthesis, the level of cellular respiration decreases linearly as a function of cell growth, suggesting that under these conditions those mitochondria which are produced are capable of a much-decreased level of respiration. Also, cytochromes *a-a<sub>3</sub>* and *b* rapidly disappear from the cell in the absence of mitochondrial protein synthesis. However, inhibition of the synthesis of mitochondrial-specific proteins appears not to decrease the number of mitochondria per cell (Galper, 1970), but to allow the accumulation in the cell of relatively empty membrane structures in which fewer cristae are



visible. Those cristae which are present are normal neither in size nor in organization.

Hence, the development of cristae with the orderly lying-down of the components of the inner membrane appears to be independent of the synthesis of outer membranes, but dependent on the presence of the products of mitochondrial protein synthesis. Further study of these proteins and development of solvent systems for their proper resolution is necessary in order more fully to understand their function.

I would like to thank Dr James E. Darnell, Jr., for his help and encouragement throughout this work, Dr Cyril L. Moore for comments and suggestions, and my wife Linda for typing the revisions of this manuscript.

This work was supported by grants from the National Institutes of Health (R01CA11159-02), National Science Foundation (GB8497), and the American Cancer Society (410-3070-2372), and Predoctoral Training Grant 5T5 GM1674 from the United States Public Health Service.

Received for publication 30 October 1972, and in revised form 13 November 1973.

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