

MODE OF MITOCHONDRIAL FORMATION IN HE_LA CELLS

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INTRODUCTION

The mode of mitochondrial formation has been so far directly investigated only in *Neurospora crassa*. By using a choline-requiring mutant of this organism, Luck (1963, 1965) has obtained evidence which strongly suggests that mitochondria are formed by growth and division of preexisting mitochondria. In *Tetrahymena pyriformis* (Parsons and Rustad, 1968), the distribution of long-term [³H]thymidine-labeled mitochondrial DNA among mitochondria during growth in unlabeled medium, as studied by autoradiography, has been found to follow a pattern compatible with this mode of mitochondrial formation. In animal cells, no evidence presently exists regarding the mode of mitochondrial formation, apart from electron micrographs of structures which have been interpreted as mitochondrial fission figures (e.g. Tandler et al., 1969; Larsen, 1970).

Both in animal cells (King et al., 1972; Storrie and Attardi, 1973) and in yeast (Linnane and Haslam, 1970) the gross formation of mitochondria has been shown to be independent of mitochondrial protein synthesis. By contrast, the formation of an active cytochrome *c* oxidase, an inner mitochondrial membrane enzyme, has been found to require the participation of the mitochondrial protein-synthesizing system in HeLa cells (Firkin and Linnane, 1968; Storrie and Attardi, 1972), as in other animal cells and in lower eucaryotic cells (for reviews see Borst, 1972; Sager, 1972).

The latter observations have suggested an experimental approach to study of the mode of mitochondrial formation in HeLa cells. Since

the number of mitochondria per cell, as recognized by morphological criteria, remains constant for up to four generations in cells growing in the presence of 40 μ g/ml chloramphenicol (a concentration sufficient to block formation of new active enzyme without any effect on the preexisting cytochrome oxidase activity [Storrie and Attardi, 1972]), a constancy in the number of cytochrome oxidase-positive mitochondrial profiles per cell section under these conditions would be in agreement with a mode of mitochondrial formation by growth and division of preexisting mitochondria. Furthermore, the distribution of preexisting cytochrome oxidase activity along the inner mitochondrial membrane, as new mitochondria are formed, should give information concerning the mode of membrane biosynthesis.

In the present work, no significant dilution in the number of cytochrome oxidase-positive organelle profiles occurred in the absence of mitochondrial protein synthesis for as many as four cell generations, while the intensity of cytochrome *c* oxidase activity along the inner mitochondrial membrane decreased uniformly. These results strongly suggest that mammalian cell mitochondria replicate by a random insertion of components into the membranes and subsequent division.

MATERIALS AND METHODS

HeLa cells were grown in modified Eagle's medium (Levintow and Darnell, 1960) with 5% calf serum, in the presence or absence of 40 μ g/ml chloramphenicol, as previously described (Storrie and Attardi, 1973). At this concentration, the drug allows at least four generations of cell growth. The cells were free of any detectable contamination by *Mycoplasma*.

A modification of the 3,3'-diaminobenzidine (DAB) reaction (Seligman et al., 1968) was used to localize cytochrome *c* oxidase activity in cell sections (Storrie and Attardi, 1973). The reaction is completely sensitive to the cytochrome *c* oxidase inhibitor, potassium cyanide. Cell sections were examined with and without staining for 30 s with lead citrate (Venable and Coggeshall, 1965) in a Philips 200 electron microscope set at 60 kV.

RESULTS

The average number per cell cross section of cytochrome *c* oxidase-positive mitochondrial profiles is not significantly altered by cell growth in the presence of 40 $\mu\text{g}/\text{ml}$ chloramphenicol for 3 days (2.7 cell generations), and is apparently decreased slightly ($\sim 25\%$) after 5 days (4.0 cell generations) of exposure to the drug (Table I). The slight decrease in number observed after 5 days of drug treatment is probably a reflection of the increased fragility of chloramphenicol-treated cells during the 3 h histochemical staining reaction. The observed number of cytochrome *c* oxidase-positive mitochondrial profiles per cell cross section in drug-treated cells is in all cases much greater than expected for a nonmitochondrial derivation of new mitochondria (Table I). This could not arise from fragmentation of mitochondria, since no change in the average size of the organelles occurs during 5 days of growth in the presence of 40 $\mu\text{g}/\text{ml}$ chloramphenicol

TABLE I

Effect of Growth of HeLa Cells in the Presence of Chloramphenicol (CAP) on the Mean Number of Cytochrome c Oxidase-Positive Mitochondrial Profiles per Cell Section

Treatment	No. cytochrome oxidase-positive mitochondrial profiles per cell section	
	Observed	Expected for non-mitochondrial origin of new mitochondria*
Control	32.4 \pm 2.5	32.4
3 days CAP (2.7 generations)	30.2 \pm 1.4	4.8
5 days CAP (4.0 generations)	23.9 \pm 1.6	2.0

Number of cells scored: control, 46; 3-day CAP, 56; 5-day CAP, 25.

* Calculated on the basis of dilution by growth of cytochrome *c* oxidase-positive mitochondrial profiles in chloramphenicol-treated cells.

(Storrie and Attardi, 1973). In both control and drug-treated cells, the incidence per cell of mitochondrial profiles stained with DAB for cytochrome oxidase activity is 20-50% higher than the number of mitochondria previously scored as organelles having a minimum of one crista (Storrie and Attardi, 1973). The difference is probably due to difficulty in scoring small mitochondria morphologically.

Fig. 1 shows mitochondria ($\times 21,000$ magnification) stained by the DAB reaction in cells grown in the absence (Fig. 1 A) or presence (Figs. 1 B, C) of 40 $\mu\text{g}/\text{ml}$ chloramphenicol. The cytochrome oxidase staining in mitochondria of control cells is fairly uniform along the periphery of the organelle and the crista projections of the inner mitochondrial membrane, though a certain patchlike distribution is recognizable, especially in the cristae. In mitochondria of cells grown for 3 or 5 days in the presence of 40 $\mu\text{g}/\text{ml}$ chloramphenicol (Figs. 1 B, C), the cytochrome oxidase staining is also fairly uniform. The progressive decrease in intensity of staining in drug-treated cells presumably reflects the 7- or 16-fold dilution in cytochrome oxidase activity per cell, which should have occurred during the 2.7 or 4.0 generations of cell growth in the presence of 40 $\mu\text{g}/\text{ml}$ chloramphenicol.

The interior of mitochondria in the chloramphenicol-treated cells appears to be devoid of staining in comparison with the mitochondria in control cells, suggesting a relative scarcity of cristae. This is in contrast to what is observed after fixation with the cross-linking agent, glutaraldehyde, where the amount of cristae per mitochondrion is the same in control and chloramphenicol-treated cells (Storrie and Attardi, 1973). Very probably, the appearance of the mitochondria stained with DAB is the result of the mild fixation of the cells with formaldehyde, which is necessary to preserve cytochrome oxidase activity. In fact, the average cross-sectional area of mitochondrial profiles in 5-day chloramphenicol-treated cells fixed with formaldehyde is 65% greater than in control cells similarly treated: expansion of the mitochondria during fixation would be expected to cause retraction of the projecting cristae.

The patchlike distribution of DAB staining for cytochrome *c* oxidase activity is much more apparent at a higher magnification ($\times 102,000$) (Fig. 2). Especially striking and regular is the patchy distribution of the DAB deposits in the whorled cristae of mitochondria of cells grown in

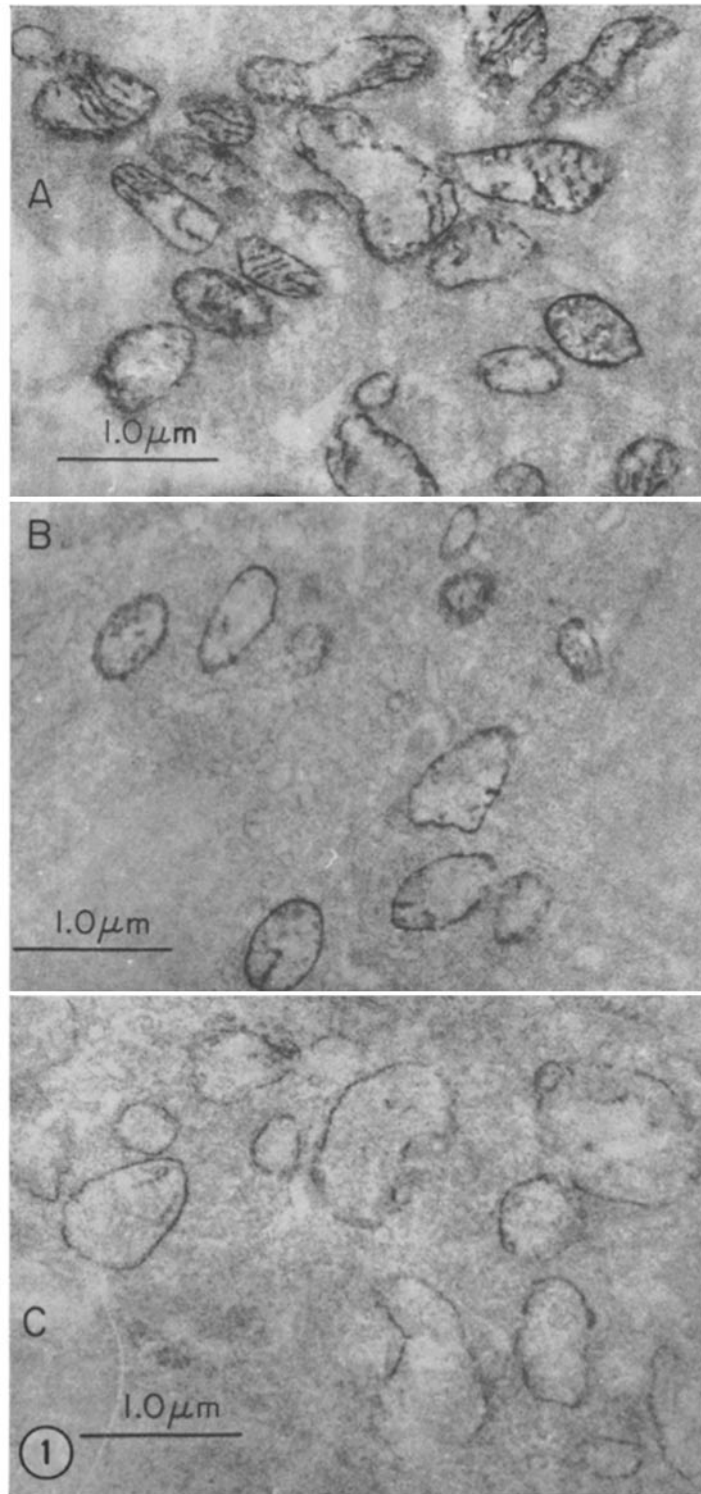


FIGURE 1 DAB-stained mitochondria in HeLa cells grown in the absence (A) or presence of 40 $\mu\text{g}/\text{ml}$ chloramphenicol for 3 days (B) or 5 days (C). $\times 21,000$.

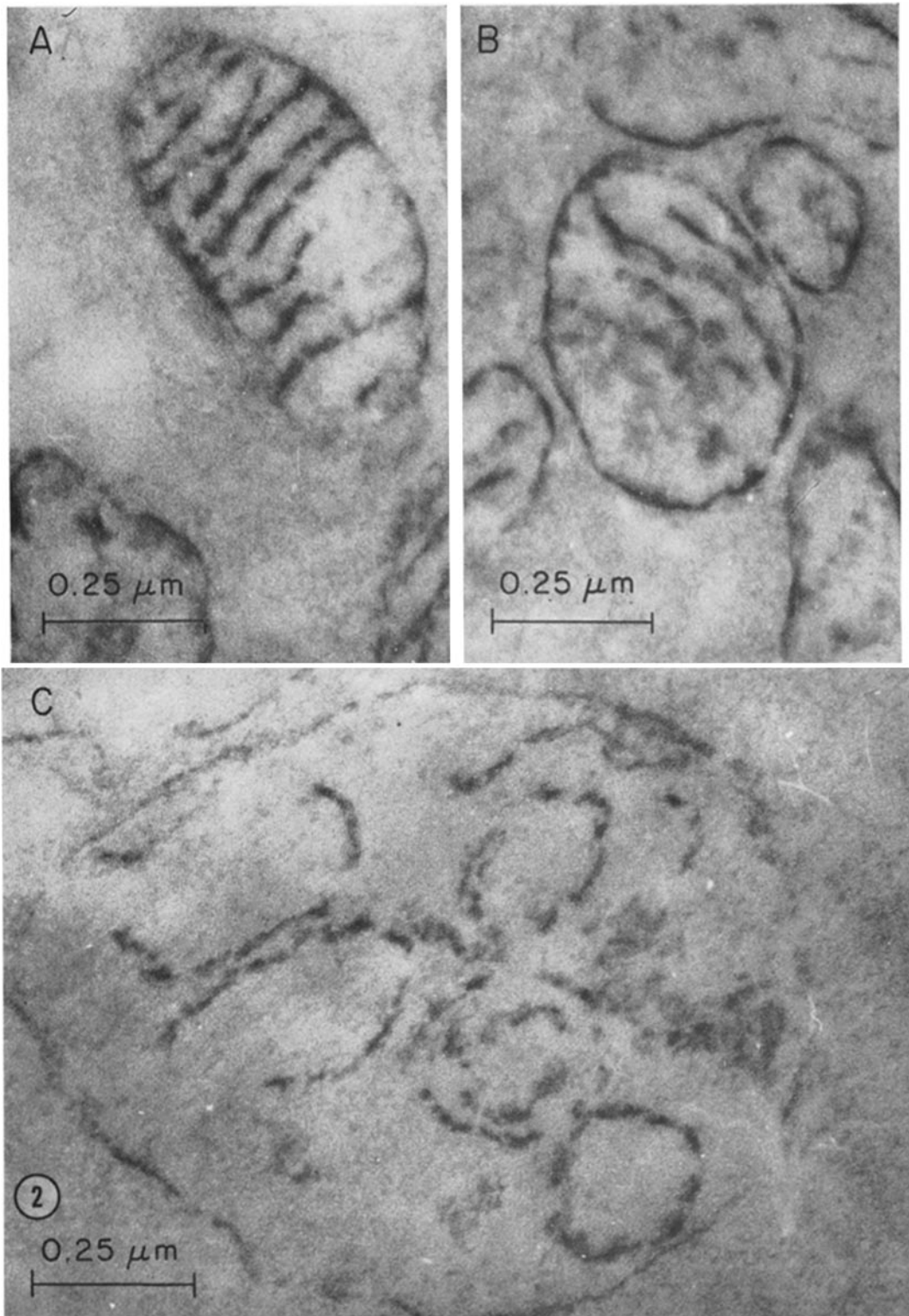


FIGURE 2 DAB-stained mitochondria in HeLa cells grown in the absence (A, B) or presence (C) of 40 $\mu\text{g}/\text{ml}$ chloramphenicol for 5 days. Note the patchiness of the DAB staining along the cristae (A) and periphery (B) of mitochondria in control cells and, especially pronounced, of DAB reaction product in the whorled cristae of a mitochondrion from a drug-treated cell (C). $\times 102,000$.

the presence of chloramphenicol for 5 days (Fig. 2 C). It should be noted that the DAB staining of the cristae appears to be more intense than that of the portions of the inner membrane which line the outer membrane, presumably because of the double membrane which constitutes these projections.

DISCUSSION

The constant number of cytochrome *c* oxidase-positive (DAB-stained) organelles per cell, in the absence of any change in the average size of the organelles (Storrie and Attardi, 1973), and the uniform decrease in DAB staining of the mitochondrial population after as many as four generations of growth of HeLa cells in the presence of chloramphenicol suggest that mammalian mitochondria, like those of *Neurospora* (Luck, 1963, 1965), arise by growth and division of preexisting mitochondria. A *de novo* origin or a derivation of mitochondria from nonmitochondrial precursors in chloramphenicol-treated cells, where new cytochrome oxidase expression is blocked but the total preexisting enzyme activity is preserved (Firkin and Linnane, 1968; Storrie and Attardi, 1972), would in fact result in a marked dilution, with cell multiplication, of the number of cytochrome oxidase-positive organelles per cell.

A formation of mitochondria independent of preexisting mitochondria could conceivably still be compatible with the finding of a uniform distribution of the preexisting cytochrome oxidase activity among mitochondria, if there were a rapid random exchange of this activity between mitochondria due to processes of mitochondrial fusion and fission (Frederic, 1958). However, the low (<1%) incidence of branched mitochondrial profiles in sections of HeLa cells (Storrie and Attardi, 1973) and the uniform DAB staining along the mitochondrial periphery and cristae in control, and especially chloramphenicol-treated cells, point to the possibility that mitochondrial fusion in HeLa cells is a rare event.

The fairly uniform decrease, as compared with control mitochondria, in the intensity of the histochemical staining of the cytochrome oxidase along the inner mitochondrial membrane in drug-treated cells most likely results from a random insertion of newly synthesized cytoplasmic proteins, individually or in complexes, into the inner mitochondrial membrane, and a conse-

quent dilution of the preexisting cytochrome oxidase activity. It is likely that also in non-drug-treated cells the inner mitochondrial membrane grows by fairly random interstitial insertion of components. The patchiness of the DAB reaction product suggests some tendency toward clustering of cytochrome oxidase and perhaps related enzymes. A patchlike distribution of cytochrome oxidase activity has also been noted by Seligman et al. (1968). Such a mode of mitochondrial membrane growth would be consistent with a fluid mosaic model of membrane structure (for review see Singer and Nicolson, 1972) and the functional requirements of the inner mitochondrial membrane.

The evidence for an interstitial mode of growth of the inner mitochondrial membrane detected here appears to be in contrast to the situation described in *Bacillus subtilis*, where a localized formation of the cytoplasmic membrane in the equatorial zone in the plane of attachment of the chromosome to the membrane has been suggested (Jacob et al., 1966). However, it should be mentioned that the validity of the latter model has been recently questioned (Mindich and Dales, 1972).

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