

## MORPHOLOGICAL STUDIES OF CELLS GROWN IN THE ABSENCE OF MITOCHONDRIAL-SPECIFIC PROTEIN SYNTHESIS

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### INTRODUCTION

The existence of autonomous protein synthesis in the mitochondria of eukaryotic cells has been well established (1, 2). The mitochondrial protein synthesis system has been characterized in unicellular organisms such as yeast, *Neurospora*, etc. (3-7). More recently, the protein synthesis system of mammalian mitochondria has been partially characterized (8-19). The active unit of this system appears to be a 55S (20) particle which occurs assembled into 95S structures (18). In mammalian cells this system produces a small number of polypeptides, perhaps six or seven (21).

Several specific inhibitors of mitochondrial-spe-

cific RNA and protein syntheses are available. Ethidium bromide inhibits mitochondrial RNA synthesis at very low concentrations. The drug has little effect on the synthesis of RNA occurring outside of mitochondria (22). It also inhibits mitochondrial DNA replication<sup>1</sup> and rapidly reduces mitochondrial protein synthesis to immeasurable levels (19). Chloramphenicol is a specific and complete inhibitor of mitochondrial protein synthesis but has little effect on other cellular synthetic processes (11, 18, 19, 23, 24).

HeLa cells in suspension culture continue to

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<sup>1</sup>Leibowitz, R. Manuscript in preparation.

grow for two generations in the presence of concentrations of ethidium bromide or chloramphenicol which almost completely inhibit mitochondrial protein synthesis (90%). The effects of such growth on mitochondrial structure and cell morphology have been examined.

#### MATERIAL AND METHODS

HeLa cells were grown in suspension culture as previously described. Ethidium bromide was present in the medium at a concentration of 0.5  $\mu\text{g}/\text{ml}$ , or chloramphenicol at 50  $\mu\text{g}/\text{ml}$ . The cells were diluted every day and the cell concentration never exceeded  $4 \times 10^6$  cells/ml.

Several fixation methods were employed, but the most satisfactory results were obtained from the following. Cells were collected by centrifugation at a speed of 800 rpm for 10 min in an International Pr-2 centrifuge (International Equipment Co., Needham Heights, Mass.) higher speeds yielded disrupted cells. The cell pellets were washed by resuspension in Earle's saline (25) and collected again. The pellets were then fixed for 1 hr in 2.5% glutaraldehyde buffered with 0.1 M cacodylic acid, and then were washed for 2 hr in 0.2 M sucrose buffered with cacodylate (26). Postfixation with 2% osmium tetroxide buffered with sodium barbital (27) was followed with a 30 min wash in 0.5% uranyl acetate (28, 29). The pellets were dehydrated in ethanol and washed for 15 min in propylene oxide (30). A mixture of two parts Epon B to one part Epon A (30) was introduced to the pellets through graded concentrations of propylene oxide. The pellets were then embedded in this same Epon mixture. Thin sections were cut on a Sorvall Porter-Blum ultramicrotome MT-2 and stained with 0.5% uranyl acetate (31) for 2.5 min, followed by lead citrate (32) for 2 min. Electron micrographs were taken with a Siemens Elmiskop I.

#### RESULTS

HeLa cells growing in suspension culture show a well-defined and reproducible growth response to either of the two agents used here. The cell generation time rises from 24 hr to greater than 48 hr. The cell number always increases by a factor of four after 4 days of incubation. After this time, growth ceases and obvious cellular disintegration sets in.

Fig. 1 shows the growth of control and ethidium bromide-treated cells. Cells grow more slowly in the presence of the inhibitor but the population does eventually increase by a factor of four. Both light microscopy and electron microscopy indicate that the drug-treated cells are about the same size as control cells, indicating an increase in cell mass

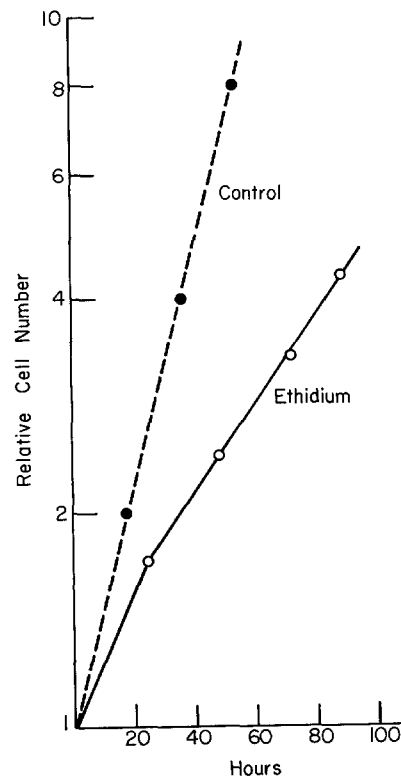


FIGURE 1 The effect of ethidium bromide on the growth of HeLa cells. Suspension cultures of HeLa cells were initially at  $2 \times 10^6$  cells/ml. Ethidium bromide was added at 0.5  $\mu\text{g}/\text{ml}$  to one culture. Cultures were diluted daily so that cell concentration never exceeded  $4 \times 10^6$  cells/ml. The relative number of cells represents cumulative growth and not the actual concentration in the culture. The same growth curve is obtained with chloramphenicol.

as well as number. Essentially the same growth curve is obtained with chloramphenicol.

Fig. 2 shows a typical section from control cells which have been stained so as to accentuate the cristae of the mitochondria. The mitochondria resemble those previously described by other investigators (33, 34). This figure may be compared to Fig. 3 which shows a section from cells incubated for 3 days in chloramphenicol. The effect on the cristae of mitochondria is quite dramatic; there are many fewer mitochondrial cristae than in control cells. A similar result has been obtained previously with the mitochondria from yeast treated with chloramphenicol (24). The inner mitochondrial membrane appears to have a drasti-

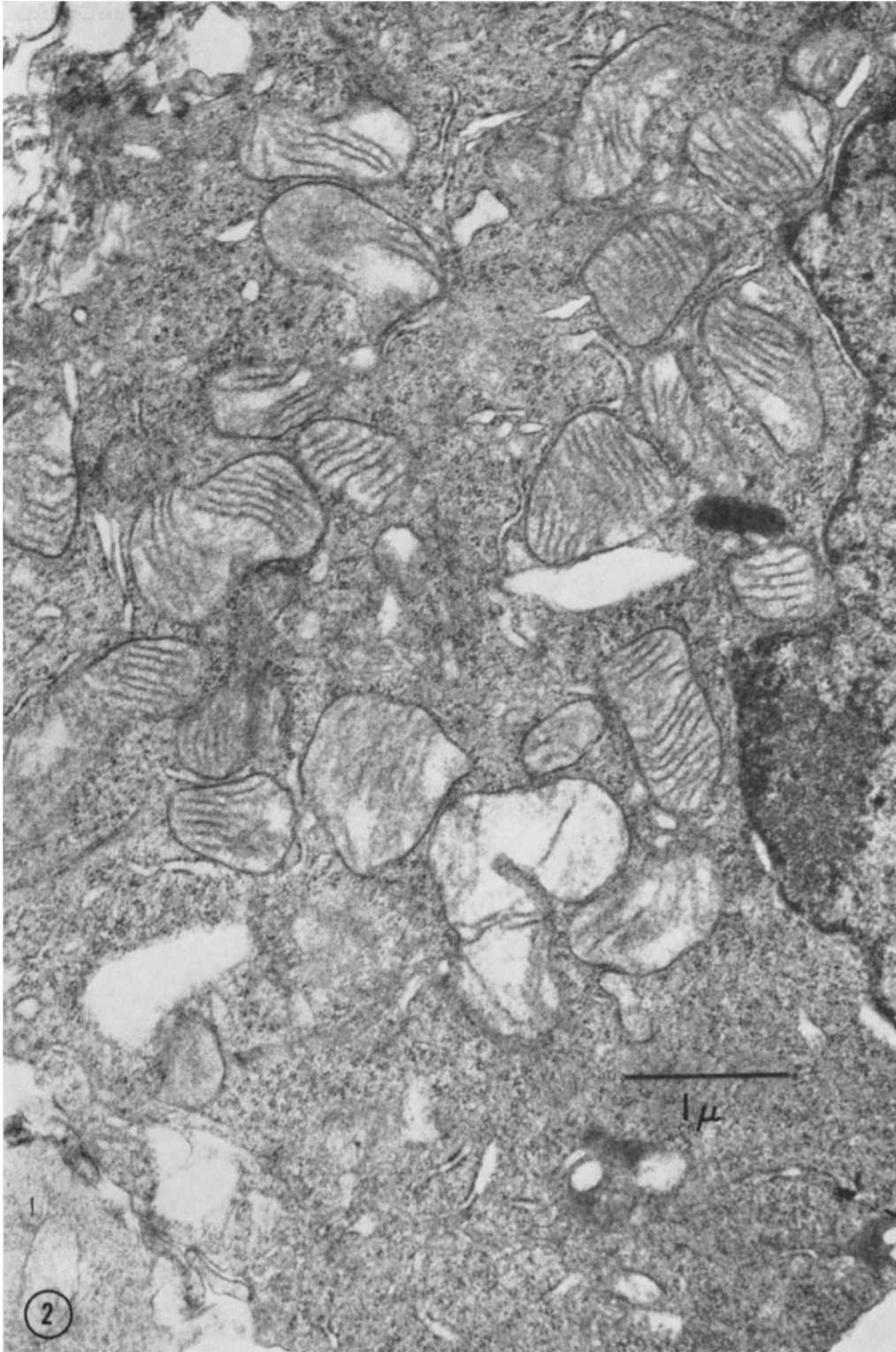


FIGURE 2 Control cells. Note the frequent occurrence of cristae in mitochondria.  $\times 24,000$ .

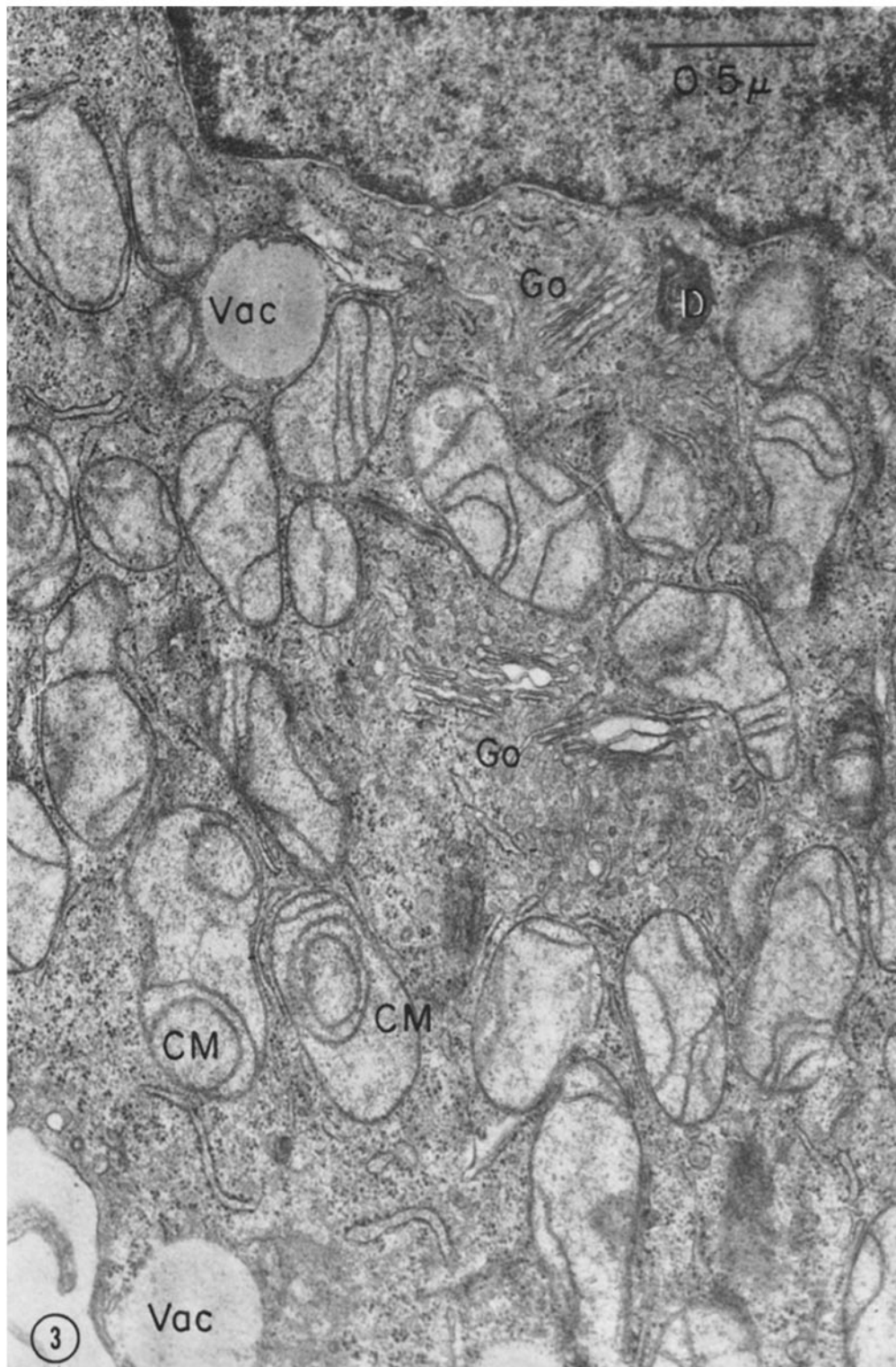


FIGURE 3 Cells incubated 3 days in chloramphenicol. Note distorted inner mitochondrial membrane and relatively scarce cristae. *CM*, circular inner mitochondrial membrane; *Vac*, vacuoles presumably containing lipids; *Go*, Golgi-like lamellar structure; *D*, dark-staining bodies.  $\times 48,000$ .

cally altered organization and it no longer forms the folds or cristae characteristic of normal mitochondria. Especially interesting is the occurrence of circular inner membranes where the structures have apparently broken loose from much of the outer mitochondrial membrane. These circular membranes are characteristic of drug-treated cells and are not seen in control cells.

The inhibition of mitochondrial-specific macromolecular metabolism eventually has significant effects on the morphology of the cell cytoplasm. Droplets apparently containing lipids appear scattered through the cytoplasm of the drug-treated cells shown in Fig. 3. These droplets are rarely seen in control cells. Also, after this long period of treatment, numerous dark-staining objects of unknown significance are found. While these objects occur within 1 day after drug treatment, they increase in number until cell growth stops.

Since cell mass has increased by a factor of four, it is possible to ask whether mitochondrial mass has been diluted by this factor. A qualitative estimate of the number of mitochondria and the contour length of the outer membrane indicates that, in fact, the mitochondrial mass is approximately the same in control and drug-treated cells. Thus the formation of organelles identifiable as mitochondria apparently does not depend upon mitochondrial-specific protein synthesis.

#### DISCUSSION

The amount of genetic information contained in the mammalian mitochondrial genome is limited and appears to code for only a few different polypeptides (21; S. Penman, unpublished). Most of the mitochondrial-specific enzymes must therefore be synthesized in the cell cytoplasm under the direction of nuclear genes. Preliminary results in this and other laboratories indicate that the synthesis of some mitochondrial-specific enzymes apparently ceases in the cell after the administration of either ethidium bromide or chloramphenicol. Presumably, the proteins synthesized in the mitochondria are necessary for the continued production or stabilization of mitochondrial proteins which are synthesized outside of the mitochondria. As the cells grow in the presence of the drug, mitochondrial activities such as cytochrome oxidase are gradually diluted out.

It appears that, despite the inhibition of formation of normal mitochondrial structure in the

presence of the two inhibitors studied here, cells continue to grow for a limited time, presumably by using the pre-existing mitochondrial proteins. An increase in cell mass by a factor of four, however, appears to exhaust this pre-existing capacity and cells thereafter enter a phase of rapid degradation.

These results suggest that the outer mitochondrial membrane can be formed in the absence of mitochondrial-specific protein synthesis since cells grown in the presence of the inhibitors appear to have an approximately normal complement of mitochondrial-like bodies with about the same surface area as in control cells. The inner mitochondrial membrane behaves quite differently. Both inhibitors cause its gradual disappearance. The inner mitochondrial membrane which remains is quite aberrant in form, and the infolding into the shape of cristae rapidly disappears. The mitochondrial protein-synthesizing system may produce some crucial proteins of the inner mitochondrial membrane or proteins necessary to correctly anchor the inner mitochondrial membrane to the outer membrane.

The appearance of lipid droplets in cells exposed to mitochondrial-specific inhibitors may reflect an interference with the fatty acid metabolism carried out by mitochondria. However, such droplets appear to be generally characteristic of pathologic states in cells and their appearance may not be a direct consequence of aberrant mitochondrial function. The appearance of Golgi-like lamellar structures, while difficult to quantitate, is a striking response to the inhibitors. Finally, the densely staining bodies observed in the cells constitute a type of structure whose origin and significance is unknown.

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