EFFECT OF CHLORAMPHENICOL ON THE ULTRASTRUCTURE OF MITOCHONDRIA IN SENSITIVE AND RESISTANT STRAINS OF HeLa

N. KISLEV, C. M. SPOLSKY, and J. M. EISENSTADT. From the Department of Human Genetics and Microbiology, Yale University School of Medicine, New Haven, Connecticut 06510. N. Kislev's present address is the Faculty of Agriculture, The Hebrew University, Rehovot, Israel.

INTRODUCTION

Ethidium bromide and chloramphenicol (CAP) are known to impair mitochondrial nucleic acid and protein synthesizing ability. These compounds also bring about alterations in the mitochondrial ultrastructure (2–9, 11). Correlation of ultrastructure with biochemical changes has been shown in both eukaryotic and prokaryotic cells grown in the presence of these inhibitors (1–9). Ultrastructural changes in mitochondria of cultured human or mouse cells have been compared to their appearance under normal conditions of growth (2–4).

The isolation and characterization of clone 296-1, a CAP-resistant mutant of HeLa S3 cells, and the study of its mitochondrial protein synthesizing ability in vitro (10), prompted us to survey its ultrastructure in the presence and absence of CAP, and to compare it to S3 cells under the same conditions. This report indicates that, in the absence of CAP, the mitochondrial morphology of this mutant is similar to that of the parental S3 cells. Although the growth rate of the mutant remains unaltered after the addition of CAP, changes occur in the mitochondrial ultrastructure, but to a much lesser extent than in sensitive cells of the parent HeLa strain. After this work was completed, similar results were reported for a mutant of *Paramecium* resistant to CAP (11).

MATERIALS AND METHODS

Cell Growth Conditions

The chloramphenicol-resistant mutant clone 296-1 was isolated from the S3 strain of HeLa cells as described by Spolsky and Eisenstadt (10). Cells were grown in suspension in modified Eagle's medium-S (MEM-S) (Grand Island Biological Co., Grand

Island, N. Y.) supplemented with 7% fetal calf serum. Monolayer cultures were grown in modified Eagle's medium-E (MEM-E) (Grand Island Biological Co.) supplemented with 10% fetal calf serum. Growth of the cultures was monitored at approximately daily intervals by counting cells in a model ZBi Coulter counter. Cells were harvested for electron microscopy after 3 days (suspension cultures), or after 8–10 days (monolayers).

Electron Microscopy

10-ml samples of cells grown in suspension in the presence or absence of 50 μg/ml CAP, containing 6–8 × 10^5 cells/ml, were added to 1 ml of cold 5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.1, and kept at 0°C for 10 min. Cells grown in monolayers were harvested by treating with 0.25% Viokase (Grand Island Biological Co.) for 10 min at 37°C and then fixed as above. All manipulations were carried out at 0°C. In each experiment S3 and mutant cells grown with or without CAP were compared. The suspensions were centrifuged at 1,000 rpm for 5 min in the International PR2 centrifuge to pellet the cells. The supernatant was replaced by fresh fixative and incubated for 2 h. The cells were then washed several times in phosphate buffer, postfixed for 1 or 1.5 h with 2% OsO_4 in the same buffer, washed and dehydrated in a graded series of ethyl alcohol, transferred into propylene oxide, and embedded in Epon 812. The sections were stained with uranyl acetate followed by lead citrate and examined in a RCA 3G or Hitachi HU-12 electron microscope.

RESULTS

Growth Curves

SUSPENSION CULTURES: Since cells of mutant 296-1 clumped and stopped growing in suspension, the experiment with suspension cultures was carried out for 3 days only. HeLa S3 cells without CAP grew at the normal exponential rate; in the presence of CAP, growth was somewhat slowed. Because of extensive clumping, accurate cell counts of mutant cultures were not possible.

MONOLAYER CULTURES: HeLa S3 cells had a generation time of 24–25 h (Fig. 1). In the presence of 50 μg/ml of chloramphenicol, S3 cells did not grow exponentially, but showed a progressively decreasing growth rate for 8–11 days, after which a rapid decrease in cell number occurred as cells began to degenerate. In experiment *a*, S3 cells were collected for ultrastructural studies at the point of maximal cell number before degeneration (Fig. 1, arrow *a*). In experiment *b*, CAP-treated

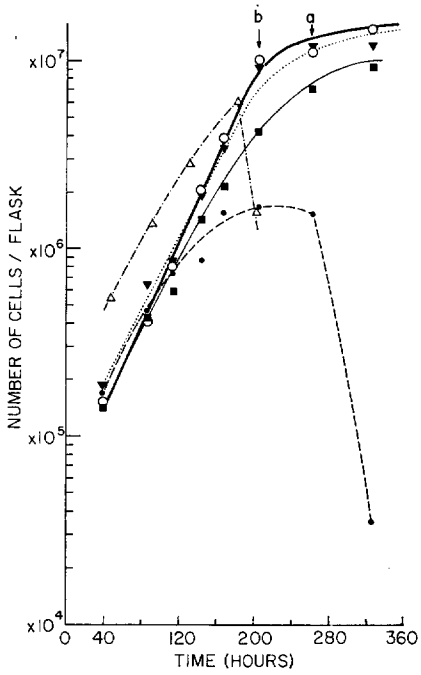

FIGURE 1 Growth curves of S3 and 296-1 with and without CAP. All cultures were grown in MEM-E + 10% calf serum. For experiment *a*, cells were harvested for electron microscopy after 264 h of growth. For experiment *b*, cells were collected after 204 h. HeLa S3 cells grown with 50 μg/ml of CAP showed a different growth pattern in the two experiments; both curves are shown. For all other cultures, growth patterns were approximately the same in both experiments. ○——○, HeLa S3, no CAP, experiment *a*. ●---●, HeLa S3 + 50 μg/ml CAP, experiment *a*. ▼ · · · ▼, 296-1, no CAP, experiment *a*. ■——■, 296-1 + 50 μg/ml CAP, experiment *a*. △–·–△, HeLa S3 + 50 μg/ml CAP, experiment *b*.

S3 cells were harvested when the decline in cell number had begun (Fig. 1, arrow *b*). As previously reported (10), mutant 296-1 has a generation time of 28–29 h. In the presence of CAP the growth rate of the mutant is essentially the same, but differs drastically from that of HeLa S3 cells grown under the same conditions. In each experiment mutant cells were collected for electron microscopy at the same time as control HeLa S3 cells (Fig. 1, arrows *a*,*b*).

Ultrastructure

In all experiments, S3 and 296-1 cells grown without CAP have the same appearance (Fig. 2).

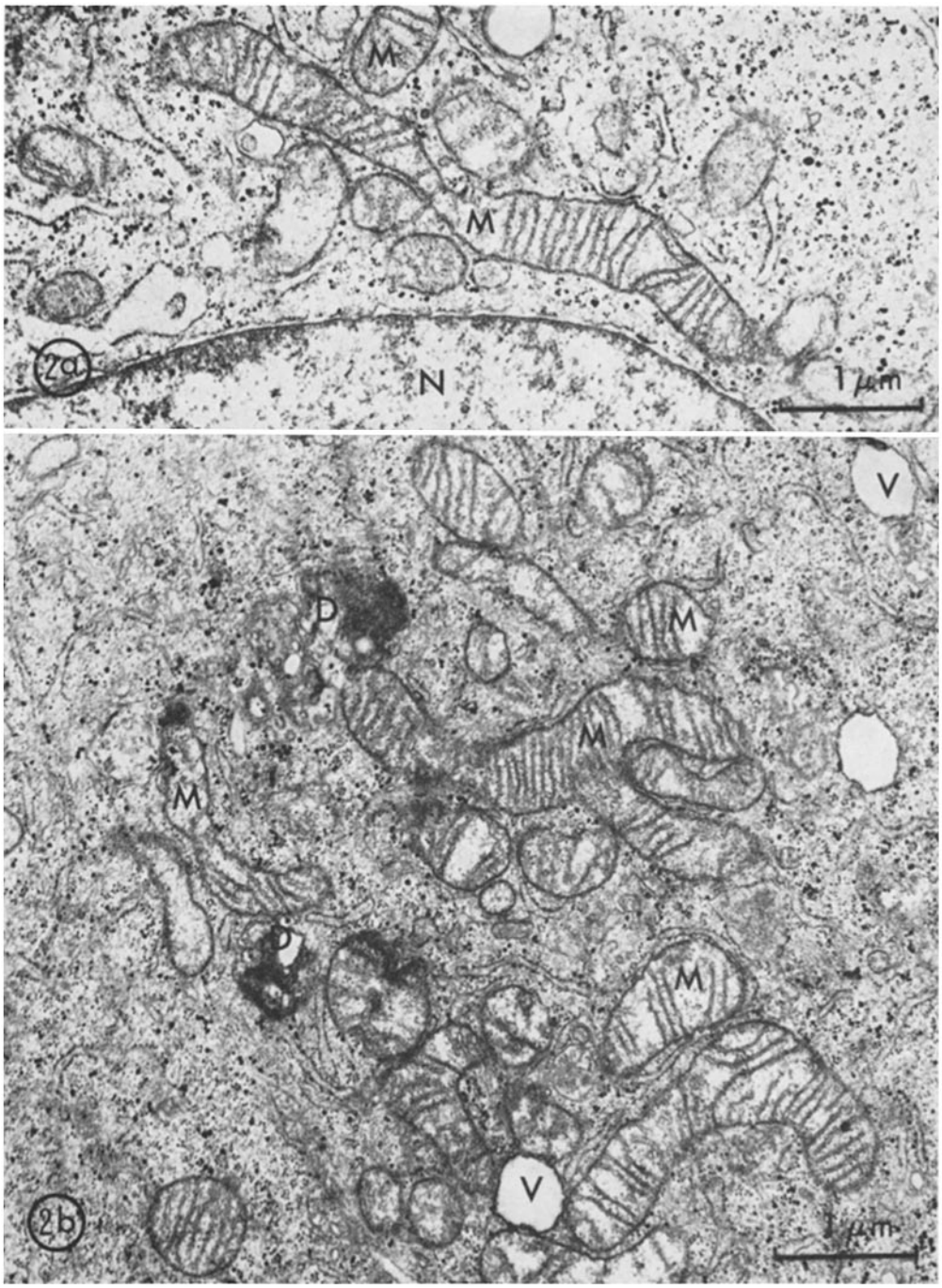

FIGURE 2 Sections of two 296-1 cells grown in the absence of chloramphenicol. Fig. 2 *a:* Profile of a long filamentous mitochondrion and a few small oblong mitochondrial profiles (*M*) are seen in the cytoplasm. Mitochondrial cristae are arranged in transverse orientation with respect to the axis of the organelle, as is common in HeLa S3 cells. *N*, part of the nucleus. Fig. 2 *b:* Cytoplasmic area of another 296-1 cell rich in mitochondria (*M*) of different sizes and shapes. Their cristae are arranged as mentioned above. A few vacuoles (*V*) and dense bodies (*D*) can be seen. × 20,000.

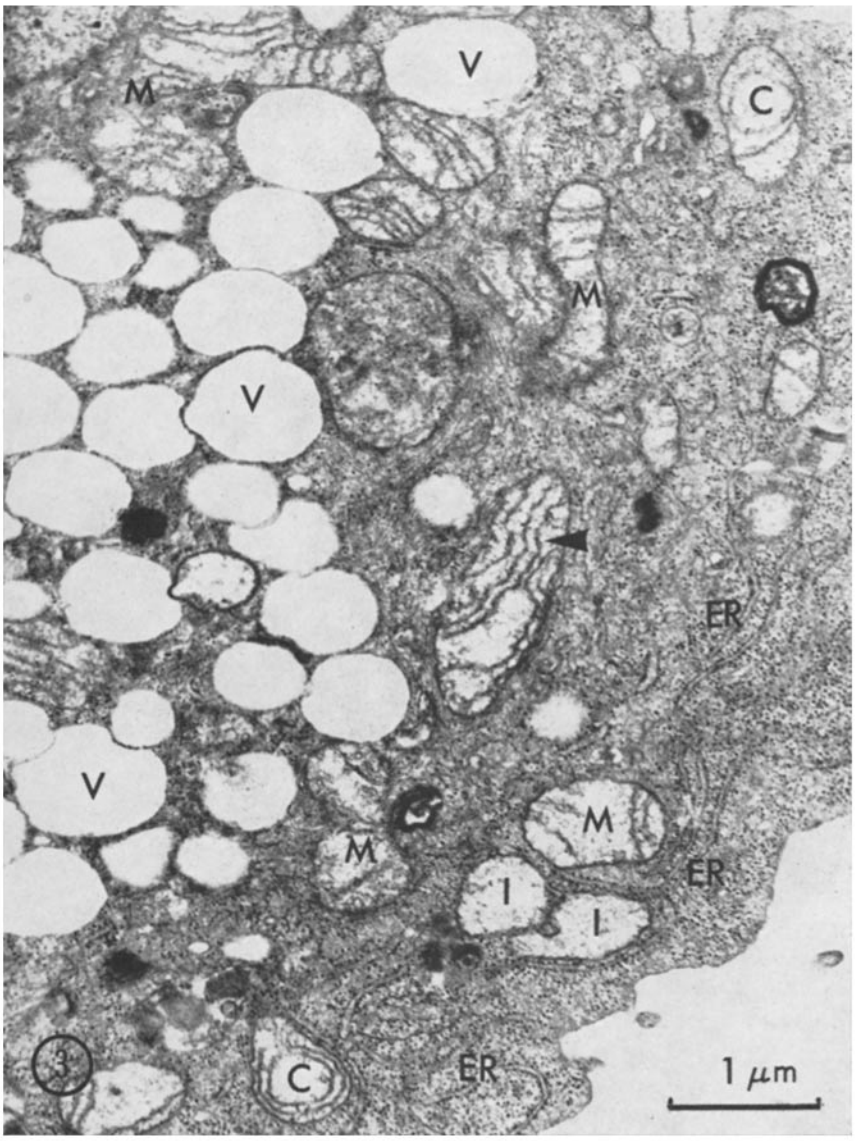

FIGURE 3 Cytoplasmic area of HeLa S3 cell growing in suspension culture in the presence of 50 μg/ml of chloramphenicol, showing mitochondria with decreased amount of cristae (*M*), changes in orientation of cristae within the organelle (arrowhead), mitochondrial profiles with circular cristae (*C*), as well as mitochondrial profiles without cristae (*I*). The cytoplasm is rich in ribosomes, rough endoplasmic reticulum (*ER*), and vacuoles (*V*). × 20,000.

Mitochondria appear filamentous or oblong with transverse cristae. It should be noted that in agreement with other work (12–14), there are structural variations among mitochondria within a cell, as well as in different cells. In most sections the density of the mitochondrial matrix was greater than that of the surrounding cytoplasm. Some mitochondria with circular cristae were observed in S3 cells, as noted by Erlandson and de Harven (12). Such cristae were also seen in 296-1 cells grown without CAP.

S3 cells grown in suspension culture containing 50 μg/ml CAP for 3 days exhibited structural changes in mitochondria (Fig. 3), namely reduc-

tion in number and changes in orientation of the cristae within the organelles, and appearance of many mitochondria with circular cristae or no cristae at all (see references 3, 4). Other alterations noted were a reduction in the electron opacity of the mitochondrial matrix, many swollen mitochondria and an increased number of vacuoles in the cytoplasm. The cytoplasm was rich in ribosomes, many of them attached to endoplasmic reticulum (Fig. 3). Under the same conditions, cells of the mutant 296-1 showed much less change in mitochondrial structure (Fig 4): in many cells mitochondria of normal appearance were found, together with a few which were partially swollen

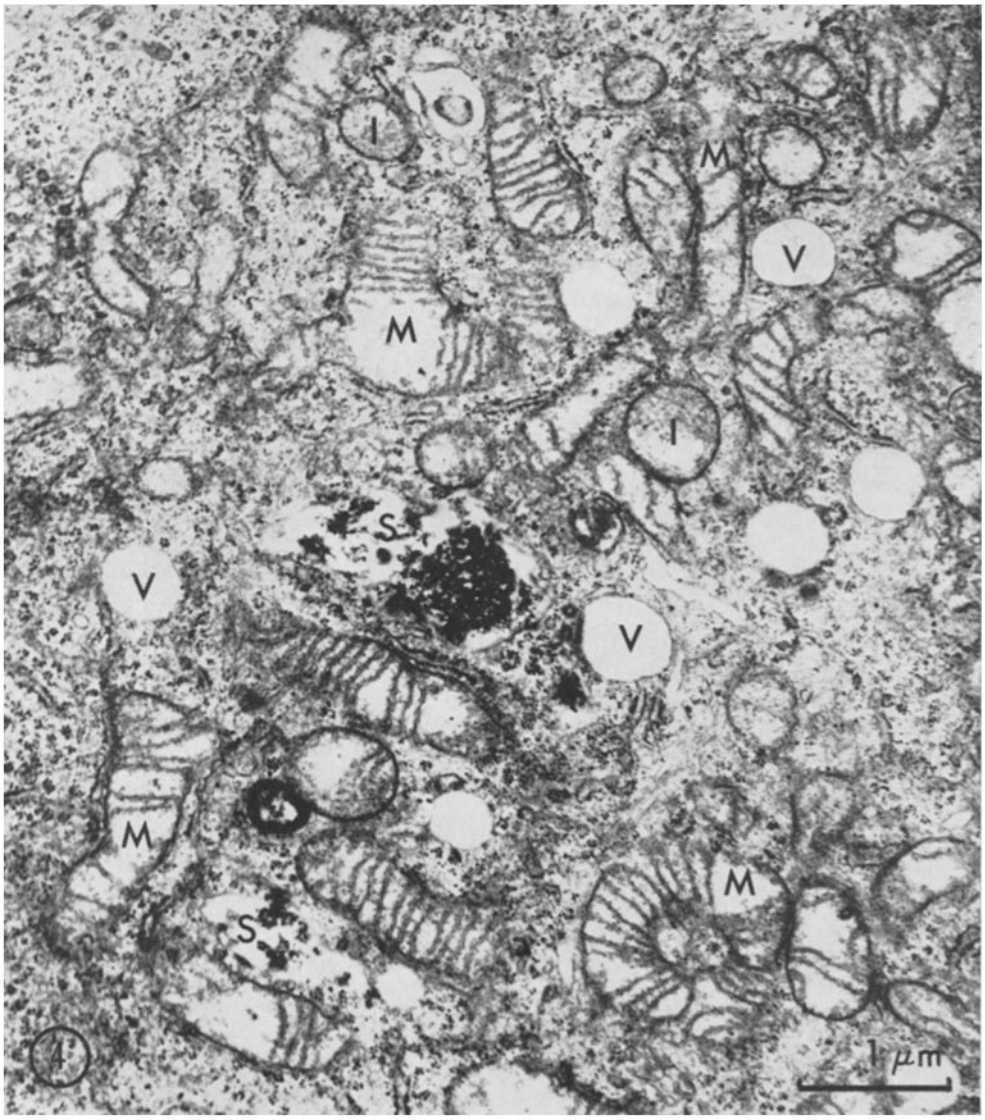

FIGURE 4 Cytoplasmic area of a mutant 296-1 cell in the presence of 50 μg/ml of chloramphenicol grown under the same experimental conditions as Fig. 3. Some mitochondrial alterations are visible when compared to mitochondrial profiles in Fig. 2 (swollen mitochondria [*M*] and mitochondria devoid of cristae [*I*]). These changes are less pronounced than those in mitochondrial profiles of HeLa S3 cells (Fig. 3). Identification as in Fig. 3. Vesicles containing dark-staining material (*S*) are also seen. *V*, vacuoles. × 20,000.

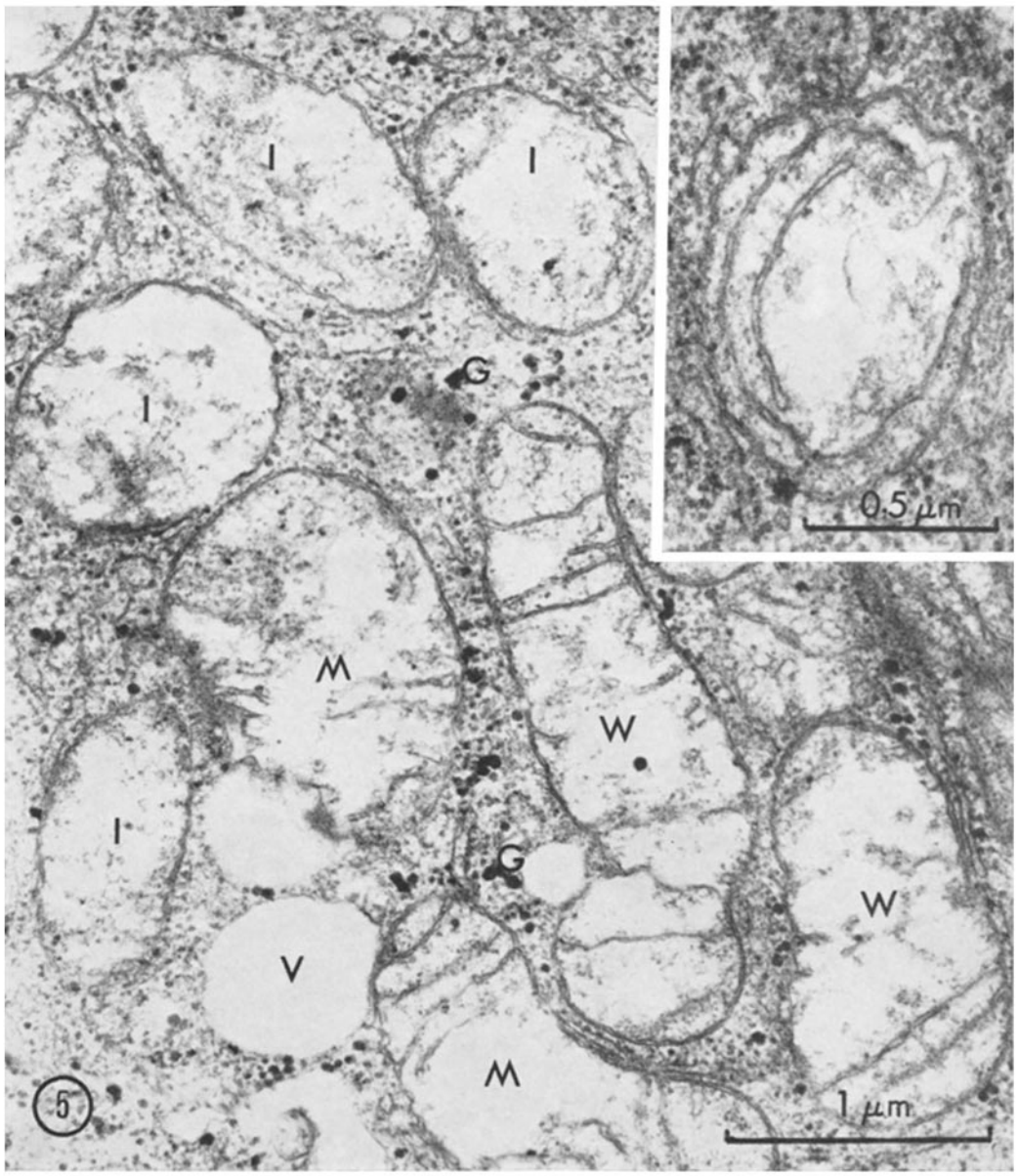

FIGURE 5 Cytoplasmic area of HeLa S3 cell grown in the presence of 50 μg/ml chloramphenicol in monolayer culture. Mitochondrial profiles are swollen (*M*), contain few cristae (*W*), or are devoid of cristae (*I*). Mitochondrial matrix is less dense than in control cells (Fig. 2). Vacuoles (*V*) and glycogen granules (*G*) are also seen. × 35,700. Inset: mitochondrial profile with circularly arranged cristae. × 51,000.

and had a reduced number of cristae. Mitochondrial changes in 296-1 were less extensive than in S3 cells.

Further studies were conducted with cells from monolayer cultures. In experiment *a*, ultrastructural changes in mitochondria of S3 cells grown in the presence of CAP were similar to those mentioned above, although the degree of change was greater and only a few mitochondria with a normal arrangement of cristae were observed (Fig. 5 and

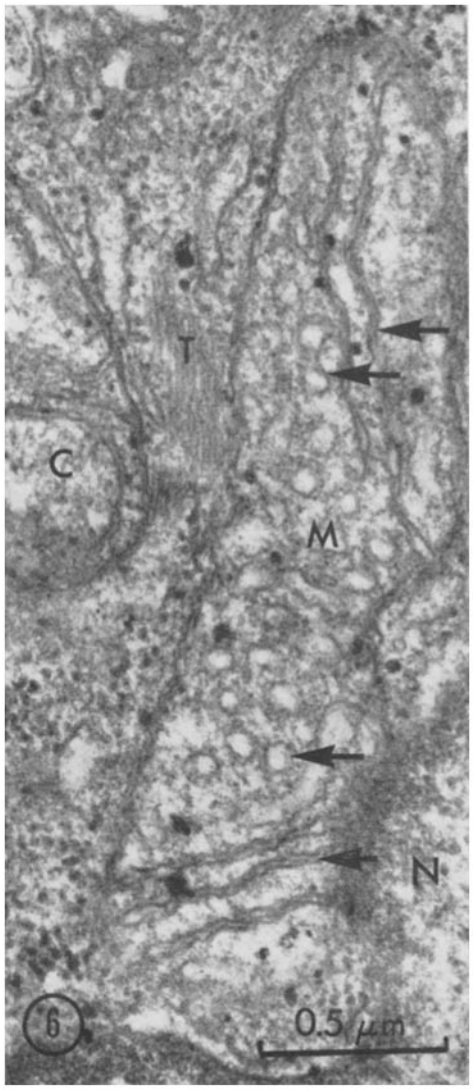

FIGURE 6 Part of HeLa S3 cell grown in the presence of chloramphenicol as in Fig. 5. Mitochondrial profile (*M*) with tubular cristae arranged in perpendicular planes (arrows). *N*, part of the nucleus; *C*, part of another mitochondrion; *T*, microtubules (also seen in control cells). × 50,000.

insert). Rarely, mitochondria with a complex arrangement of cristae were seen (Fig. 6). In cells of mutant 296-1 many mitochondria with normal appearance were found (Fig. 7). There was less swelling of the mitochondria and a smaller decrease in the number of cristae. There were also variations among cells in the same section, a few resembling untreated cells in appearance.

In experiment *b* most S3 cells grown in the presence of CAP (electron micrographs not included) were already in a condition of advanced disintegration, in which most mitochondria had the appearance of empty double-layered vacuoles. Other cell organelles, such as endoplasmic reticulum and the Golgi apparatus, were swollen and the cytoplasm appeared distorted. The nuclei had a condensed area of DNA in their periphery, and vacuoles appeared between the nuclear envelope and the cytoplasm. Cells not in this condition contained cytoplasm similar to that in untreated cells and swollen mitochondria with a few distorted cristae or none at all. Only a few mitochondria with normal transverse cristae were observed. Mitochondrial profiles of 296-1 cells were similar to those observed in experiment *a*. In some cells many mitochondria were swollen and contained few or distorted cristae resembling those reported by Lenk and Penman (3). However, in those cells the cytoplasm also looked different from that in normal cells. (Variation in the structure of mitochondria within the same cell and among different cells was observed.)

DISCUSSION

In agreement with previous observations (3, 4), we have found that HeLa S3 cells grown in the presence of CAP contain mitochondria exhibiting gross morphological changes. Virtually all the mitochondria of sensitive cells show a disturbance of normal structure of one kind or another. It should be noted that the outer mitochondrial membrane remains even where cristae show maximum morphological change.

By contrast, cells of the CAP-resistant mutant, 296-1, grow equally well in the presence or absence of CAP. Such cells exhibit minimal changes in mitochondrial structure. We have also found that the mutant 296-1 grown for almost 1 yr in the presence of CAP (10) is capable of reverting to a normal ultrastructure within 3 days in suspension culture without CAP. The mutation is a stable one: cells grown in the absence of CAP for approximately 250 generations are still resistant to the drug.

At this point we are not absolutely sure of the site of action of the mutation. There is evidence in yeast cells (15) of CAP-resistant mutations which are cytoplasmically inherited and which affect mitochondrial permeability. Other yeast strains have cytoplasmically inherited erythromycin re-

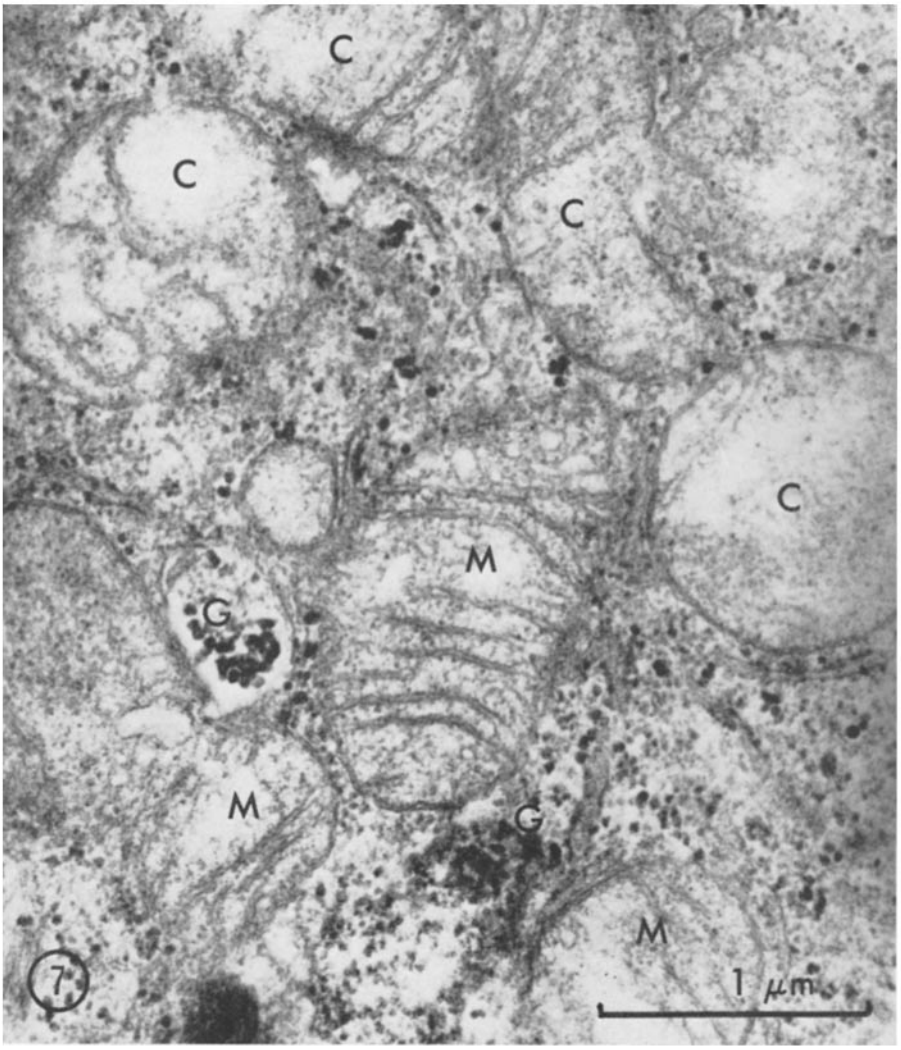

FIGURE 7 Cytoplasmic area of chloramphenicol-resistant 296-1 cell growing under the same experimental conditions as in Figs. 5 and 6. Some mitochondrial alterations are visible (decrease in number of cristae, and changes in their orientation [*C*]). Mitochondria with normal appearance (*M*) can also be seen. The cytoplasm is rich in ribosomes. *G*, glycogen granules. × 35,700.

sistance, resulting from a change in permeability or alteration of the protein synthetic system within the mitochondria (15, 16).

That the mitochondria of resistant cells show some morphological changes in the presence of CAP indicates that the CAP is entering the cells and affecting the mitochondria. This is in contrast to observations on viable cells obtained after prolonged growth of sensitive S3 cells in the antibiotic (2, 4); these cells contained mitochondria with a normal ultrastructure. Biochemical studies of the mutant 296-1 confirm ultrastructural evidence that the resistance is intramitochondrial. Mitochondria isolated from the resistant mutant 296-1 actively incorporate amino acids into protein in vitro in the presence of CAP, whereas HeLa S3 mitochondria are inhibited (10). 296-1 mitochondria remain resistant to CAP inhibition when treated with Triton X-100, a nonionic detergent which disrupts the mitochondrial membrane structure (10).

Taken together, these two pieces of evidence

make it unlikely that the mutation in strain 296-1 is one affecting permeability to CAP of either cell or mitochondrial membranes. It suggests an intramitochondrial location of the mutant gene product, either in the mitochondrial protein synthesizing system itself or in a CAP-modifying enzyme activity.

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