THE EFFECTS OF ERYTHROMYCIN AND CHLORAMPHENICOL ON THE ULTRASTRUCTURE OF MITOCHONDRIA IN SENSITIVE AND RESISTANT STRAINS OF *PARAMECIUM*

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ABSTRACT

The effects on cell structure of 12 hr to 6 days of exposure to erythromycin or chloramphenicol, two antibiotics known to inhibit specifically the mitochondrial protein synthesizing system, have been studied in the ciliate *Paramecium aurelia*. A wild type strain (sensitive to both antibiotics) and three mutant strains carrying cytoplasmically inherited mutations conferring resistance to one or the other antibiotic have been used In sensitive cells both antibiotics lead to a progressive and profound alteration of mitochondrial structure evidenced by an elongation of the organelle, a considerable decrease in the number of cristae, and the appearance of some abnormal lamellar cristae and of rigid plates of periodic structure. The modifications of cell structure, then, are mainly restricted to mitochondrial cristae. The three resistant mutants studied, on the contrary, retain normal or nearly normal mitochondrial structure in the presence of the antibiotic to which they are resistant. This fact is in good agreement with the postulated location in the mitochondrial DNA of the resistance mutations studied. The results are discussed in the light of present knowledge concerning the function of the mitochondrial protein-synthesizing system.

INTRODUCTION

It is now well established that, in eukaryotic cells, antibacterial antibiotics such as erythromycin and chloramphenicol block specifically the mitochondrial protein-synthesizing system without altering the cytoplasmic one (Mager, 1960; Kroon, 1965, Wintersberger, 1965; Clark-Walker and Linnane, 1966, 1967; Firkin and Linnane, 1968; Lamb et al , 1968; Perlman and Penman, 1970). The block leads to a series of biochemical and cytological consequences that have been studied in several organisms and that consist mainly of the loss of several insoluble mitochondrial enzymes (cytochromes $a-a_3$, b, c_1) and of the disappearance of cristae (Clark-Walker and Linnane, 1967; Kellerman et al., 1969; Mason et al., 1970; Smith-

Johannsen and Gibbs, 1970; Mahler et al., 1971; Lenk and Penman, 1971).

Mutants resistant to these antibiotics have been isolated in yeast and most of them have been shown to be cytoplasmically inherited, the mutation being located in the mitochondrial DNA (Thomas and Wilkie, 1968; Linnane et al , 1968; Coen et al , 1970). A similar situation has recently been described in *Paramecium aurelia*, which is a strict aerobe. Erythromycin and chloramphenicol block *Paramecium* growth at relatively low concentrations (100–200 μg/ml) and lead to cell death. Resistant mutants have been obtained and their cytoplasmic inheritance demonstrated (Beale, 1969; Adoutte and Beisson, 1970, 1972). Other

evidence in favor of the location of these mutations in the mitochondrial DNA has been provided by Beale et al. (1972).

It was therefore interesting to study the structure of the mitochondria of both sensitive and resistant strains in the presence of antibiotics

This paper describes the effects of erythromycin and chloramphenicol on the ultrastructure of mitochondria of sensitive strains and of several resistant mutants of *Paramecium aurelia* The ultrastructure of mitochondria of sensitive cells displays a series of striking alterations which may provide information on the contribution of the mitochondrial protein-synthesizing system to the biogenesis of mitochondria. The resistant strains retain unaltered or only slightly altered mitochondria in the presence of the antibiotics. This is in good agreement with the postulated location of these mutations in mitochondrial DNA.[1]

MATERIALS AND METHODS

Strains

The wild type strain used in these experiments originated from stock d_{4-2}, syngen 4 of *Paramecium aurelia*. Three resistant mutants derived from this wild type strain have also been used. The isolation, characterization, and genetic analysis of these mutants have been described in detail (Adoutte and Beisson, 1970, 1972). Their characteristics are the following:

E_1^R is a weakly erythromycin-resistant mutant its growth in 100–200 μg/ml erythromycin is nearly normal, but slow in 400 μg/ml;

E_{102}^R is a strongly erythromycin-resistant mutant: it grows normally in 400 μg/ml erythromycin;

C_2^R is a chloramphenicol-resistant mutant: it grows slowly in 200 μg/ml chloramphenicol.

Media and Culture Conditions

Paramecia are grown according to the usual techniques (Sonneborn, 1970). The media used are the following:

nonselective medium: the usual medium is a "Scotch Grass" infusion bacterized with *Aerobacter aerogenes*, in this medium, all the strains multiply equally well at the rate of four to five fissions per day at 27°C;

selective media: to the usual bacterized medium, a concentrated solution of one of the two antibiotics is added just before utilization so as to

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[1] An abstract of this work has been published in *J. Microsc.*, 1971, 11:24.

reach a final concentration of 200 μg/ml for chloramphenicol and 400 μg/ml for erythromycin, at these concentrations of antibiotics, the growth of the sensitive cells is blocked after one or two residual fissions, but the cells can survive up to 10 days without further division

The experimental procedure is the following. About 2500 log-phase cells are transferred into 250 ml of selective medium or nonselective medium for the controls. After 12 hr, 18 hr, 1, 2, 3, 4, 5, or 6 days at 27°C, the cells are counted in order to estimate the rate of growth, centrifuged, and fixed.

Electron Microscopy

The loose pellet of centrifuged cells is fixed in 0 25% glutaraldehyde (Eastman Kodak Co., Rochester, N. Y.) in 0.05 M cacodylate buffer, pH 7–7.2, for 30 mm, rapidly washed in the buffer, and postfixed in 2% osmium tetroxide in the same buffer. After 1 hr in the second fixative, the cells are washed in 0.05 M phosphate buffer, pH 7–7.2, preembedded in a fibrin clot according to Charret and Fauré-Fremiet (1967), dehydrated in alcohol, propylene oxide, and embedded in Epon according to Luft (1961). After sectioning on a Sorvall MT-1 ultramicrotome, the sections are stained with saturated uranyl acetate followed by lead citrate, carbon coated, and examined with a Siemens electron microscope, model 1A Elmiskop

RESULTS

Effects of Erythromycin and Chloramphenicol on Sensitive Cells

A number of works have established that ciliates have rather large, oval, or slightly elongated mitochondria provided with numerous curved tubular cristae (for general literature, see Jurand and Selman, 1969). The wild type (antibiotic sensitive) strain of *P aurelia* used in this study conforms to these features, as can be seen in Fig. 1

Counts made before each fixation indicate that sensitive cells placed in the presence of the antibiotic undergo one or two residual fissions, on the average. These fissions have already occurred at the time of the first sampling (18 hr). After that time, the number of cells remains constant; no mortality occurs, although the appearance and behaviour of the cells progressively decline. they become dark and swim more slowly, becoming nearly immobile by the fourth day

Fig 2 gives a general picture of the effects of erythromycin on these cells after 2 days in presence of the antibiotic. Quite similar effects are obtained

with chloramphenicol The most conspicuous modification induced by the antibiotic is a markedly reduced mean diameter and an increased length of most of the mitochondria Other types of structural alterations are observed, examples of which are presented at higher magnification in Figs 5–10. They can be briefly described as.

(a) a notable decrease in the number of cristae, exhibited by most of the mitochondria (Figs 2, 5);

(b) a collapsed appearance of some mitochondria; these mitochondria are completely flattened, having almost nothing left but their envelope and very little matrix (Fig 5);

(c) a repetitive disposition of wavy tubular cristae, occurring in some mitochondria (Figs. 6, 7);

(d) the existence, in some mitochondria, of membrane pairs applied against the inside aspect of the envelope, these membrane pairs look like lamellar cristae (Figs 5, 6, 8), two, three, or more can be piled up, parallel to each other and to the mitochondrial envelope,

(e) the existence, in some mitochondria, of one or two atypical cristae, in the shape of plates of rigid appearance 250 A thick (Figs 5, 6, 8–10), similar to those described by Newcomb et al (1968) in bean root mitochondria, like those, they are quintuple-layered, exhibit a periodic structure, and are, in some cases, continuous with the inner membrane of the mitochondrial envelope (Fig 6); they are also often seen in continuity with lamellar cristae described under d (Figs 5, 6) These striking objects have not been observed before in cells treated with the same antibiotics, they deserve a more extensive analysis

It must be noted that in all these abnormal mitochondria, even in those completely lacking cristae, both outer and inner mitochondrial membranes persist Furthermore, no other cellular structure is apparently modified: cell cortex, trichocysts, and nuclei appear normal. The effects of the antibiotics seem therefore *restricted to the mitochondrial cristae*

There is, however, a progressive decrease in the number of glycogen granules in the cytoplasmic background and the appearance, by the fifth day, of amorphous, rounded bodies about the size of a mitochondrion, electron-opaque, and not membrane limited (Fig. 5). Analogous bodies also have been observed by Lenk and Penman (1971) in HeLa cells treated with chloramphenicol and by Ben Shaul and Markus (1969) in *Euglena* treated with the same antibiotic. In both cases, they have been assumed to be lipid in nature Up to now, we have neither been able to prove nor disprove this point

There is no definite evidence yet of a sequential relationship between the various alterations observed. However, a few facts stand out when one compares cells fixed at various times of incubation with the antibiotic Up to the first 24 hr, the only modifications observed are (a) the decreased number of cristae and (b) the appearance of the membrane pairs parallel to the mitochondrial envelope. By the 24th hour, the wavy pattern develops. Finally, the first plates are observed around the 48th hour. The number of cristae decreases and the frequency of the plates increases as the exposure to the antibiotic is prolonged

The detailed examination of the modifications as a function of time suggests that the membrane pairs develop at the expense of cristae and that plates result from a progressive stiffening of membrane pairs. Indeed, plates are quite often observed in continuity with membrane pairs (Figs. 5, 6) and sometimes appear in continuity with the inner membrane of the mitochondrial envelope (Fig 6)

Finally, it must be pointed out that until the latest stages observed (6 days), all the cells remain capable of resuming growth when transferred to nonselective medium (100 tested cells in two experiments) after a lag of 24–48 hr The effects of the antibiotics are therefore reversible even at

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FIGURE 1 Cytoplasmic area of a wild type cell in the absence of antibiotic, showing the normal appearance of *Paramecium* mitochondria These mitochondria are rounded or slightly elongated, with numerous, irregularly curved, tubular cristae and little matrix Tt, trichocyst tip; Tb, trichocyst body; the dark dots in the cytoplasmic background are glycogen granules. × 30,000

FIGURE 2 Cytoplasmic area of a wild type cell after 2 days of exposure to 400 μg/ml erythromycin, showing a number of modifications of the mitochondria. By comparison with the preceding figure at the same enlargement, the mitochondria appear smaller in diameter, more elongated, with reduced numbers of cristae and a denser matrix. Some are devoid of cristae (m); others have regularly wavy cristae (W) or lamellar cristae (short arrow), or a rigid plate (long arrow). × 30,000.
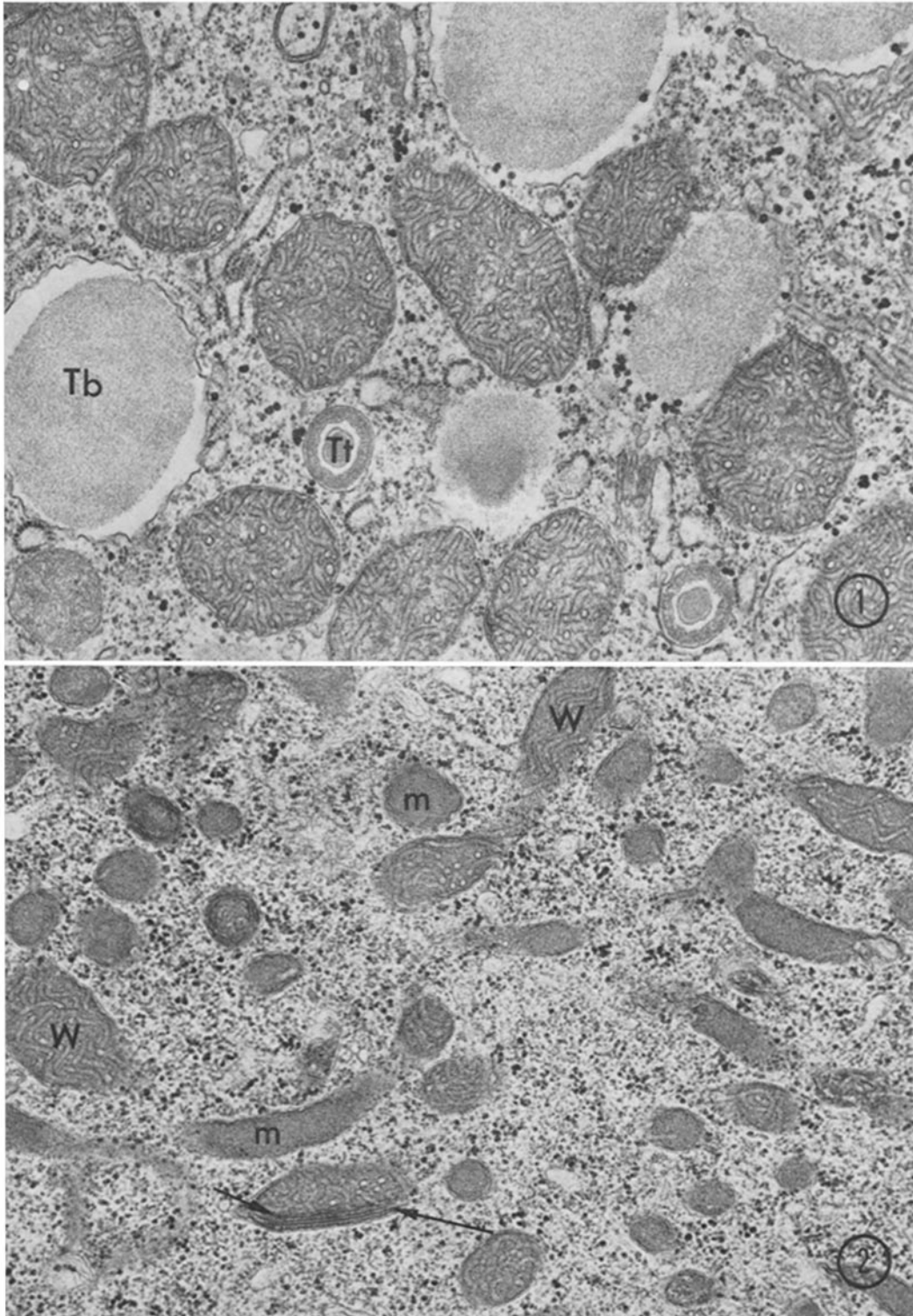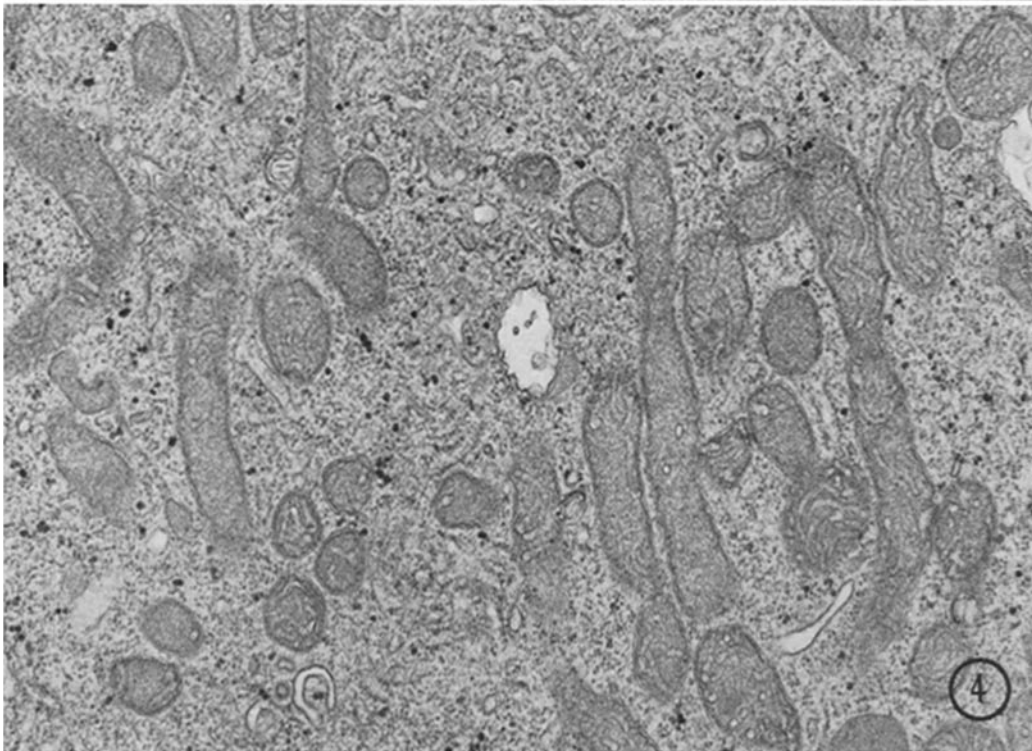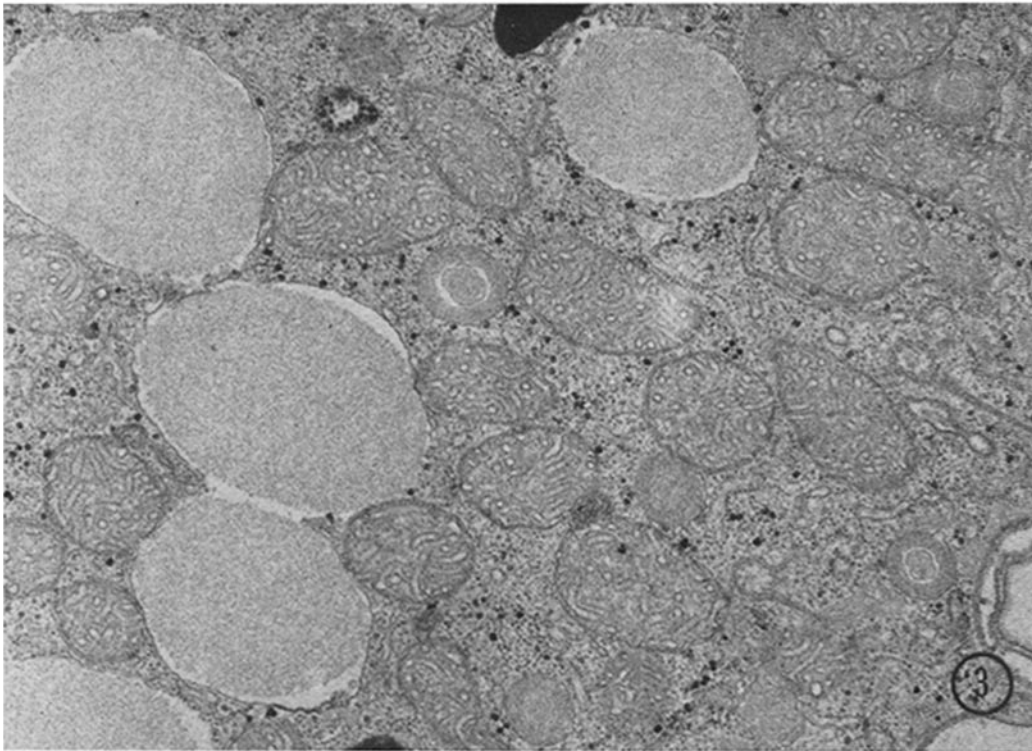
FIGURE 3   Cytoplasmic area of the erythromycin-resistant mutant E_{102}^R after 6 days of exposure to 400 µg/ml erythromycin. Mitochondrial structure appears quite normal. This mutant is strongly resistant. × 30,000.

FIGURE 4   Cytoplasmic area of the chloramphenicol-resistant mutant C_2^R after 3 days of exposure to 200 µg/ml chloramphenicol. Some mitochondrial alterations are visible (elongation, disappearance of cristae) but are less pronounced than in the wild type in Fig 2; the plates do not appear. This mutant is only weakly resistant. × 30,000.
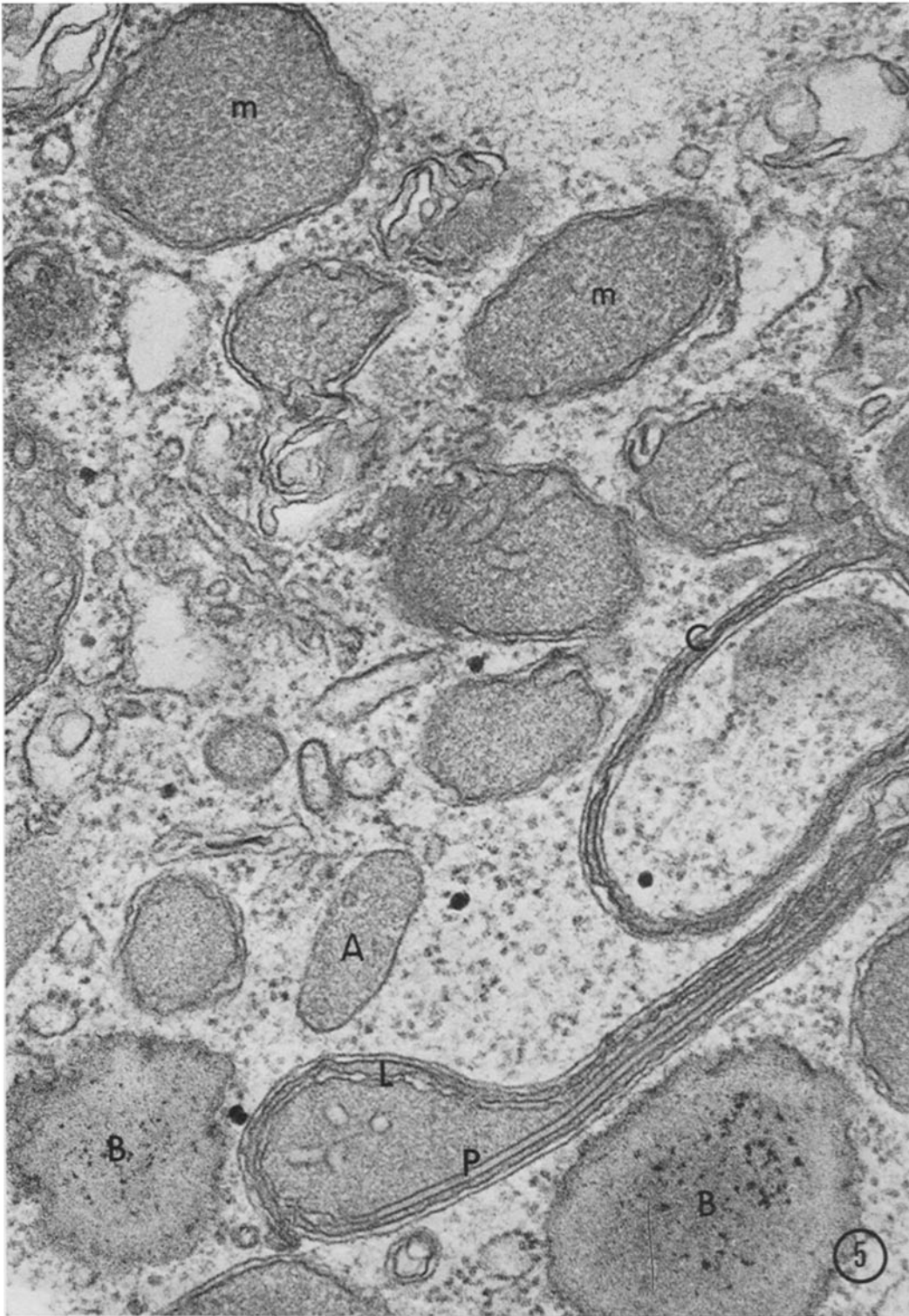
FIGURE 5 Some characteristic mitochondrial alterations in a wild type cell after 6 days of exposure to 400 μg/ml erythromycin. Some mitochondrial sections do not show any cristae (m) while the others in the field are nearly devoid of normal tubular cristae. One mitochondrion (C) is collapsed, being reduced to a flattened body; another is very elongated and contains a lamellar crista (L) continuous with a rigid plate (P). B, nonmembrane-limited amorphous bodies; A, peroxisome. × 80,000.

FIGURE 6 Mitochondrial alterations in a wild type cell after 6 days of exposure to 200 μg/ml chloramphenicol. These alterations are quite similar to those provoked by erythromycin (Figs 2 and 5) · disappearance of normal tubular cristae (m), appearance of wavy tubular cristae in register (W), of lamellar cristae (L), and of a rigid plate (P) in continuity with the inner mitochondrial membrane and with a lamellar crista (arrows). × 84,000

stages at which almost all mitochondria are severely altered.

Effects of Erythromycin and Chloramphenicol on Resistant Mutants

Three antibiotic-resistant mutants (two erythromycin-resistant, E_1^R and E_{102}^R, and one chloramphenicol-resistant, C_2^R) were examined both in nonselective and in selective medium after various periods of exposure In nonselective medium where all three mutants grow exactly like wild type cells, their mitochondria are normal and un-

distinguishable from those of wild type cells in the same conditions. In selective medium, mitochondrial structure remains normal, or more or less altered, depending on the mutant. E_{102}^R remains completely unaltered (Fig 3); E_1^R is very slightly affected and shows only a reduced number of cristae, C_2^R is markedly affected by chloramphenicol (Fig 4), but much less than the sensitive cells placed in the same conditions: an important number of cristae are still present and neither plates nor amorphous bodies are ever observed

Thus, the resistance phenotype, defined by the ability of the cells to grow in the presence of the
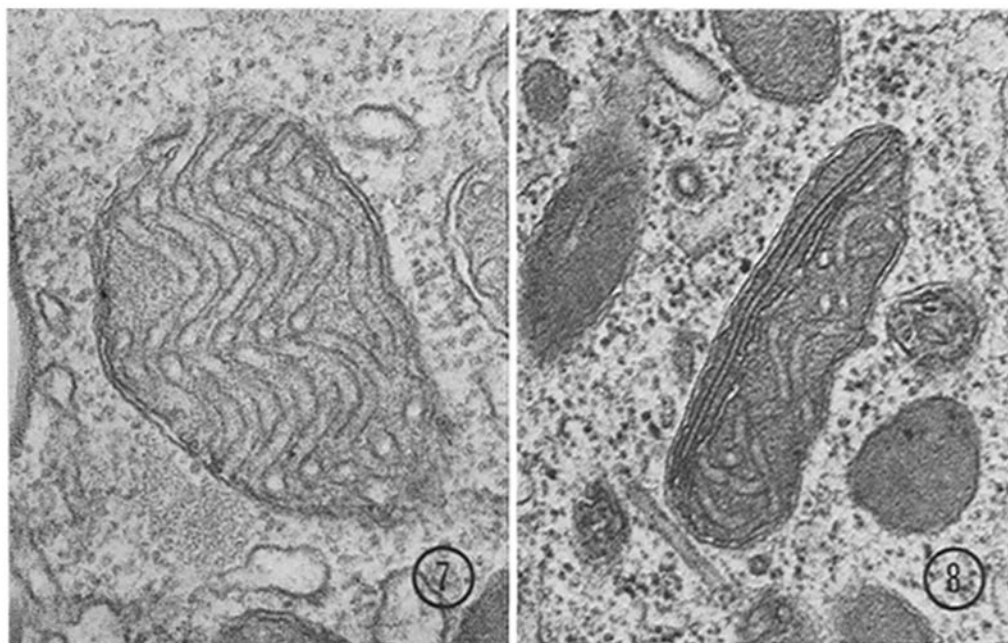
FIGURES 7 and 8   Mitochondria of wild type cells after 5 days of exposure to 200 μg/ml chloramphenicol. Fig 7 the tubular wavy cristae are arranged in register. × 80,000   Fig 8 This mitochondrion contains wavy cristae, almost in register, a lamellar crista, and a rigid plate × 60,000.

antibiotic, is paralleled by a clear-cut preservation of the mitochondrial structure in conditions in which the mitochondria of sensitive cells are considerably altered It is worth pointing out that the degree of preservation of mitochondrial structure of the resistant mutants in the presence of the antibiotic shows a satisfactory correlation with the level of resistance which characterizes each mutant While E_{102}^R is highly resistant and grows equally well in selective and in nonselective media, E_1^R is slightly less resistant and grows in 400 μg/ml erythromycin at a slower rate than in nonselective medium, and C_2^R multiplies in 200 μg/ml chloramphenicol at a markedly reduced rate (two to three fissions per day instead of four to five fissions per day in nonselective medium)

DISCUSSION

The results reported here show that erythromycin and chloramphenicol have striking effects on mitochondrial structure in sensitive cells of Paramecium while resistant mutants are not at all, or much less, affected. These results are in good agreement with those of Knowles (1971) who studied the effects of erythromycin on sensitive

Paramecium especially after microinjection of mitochondria isolated from a resistant mutant.

Part of the explanation of the various alterations observed, particularly the loss of cristae, most probably lies in the known primary effect of these antibiotics, i.e., the blockage of mitochondrial protein synthesis This blockage is known to result in the disappearance of a series of mitochondrial enzymes, as has been shown in a variety of organisms ranging from yeasts to mammals (Clark-Walker and Linnane, 1966, 1967; Firkin and Linnane, 1968, Kellerman et al , 1969) and including the ciliate Tetrahymena (Mason et al , 1970) Assuming that the antibiotics have the same effects on Paramecia, the results presented here can be explained in at least two ways. The antibiotic could either block the translation, within the mitochondria, of the messenger RNAs corresponding to the lost enzymatic proteins, thus depriving the cristae of some of their constitutive elements, or, if these enzymes are synthesized in the cytoplasm, block the synthesis of one or more protein(s) synthesized in the mitochondria which are necessary for a correct positioning of the enzymes and building of the cristae The latter hypothesis is the more likely,
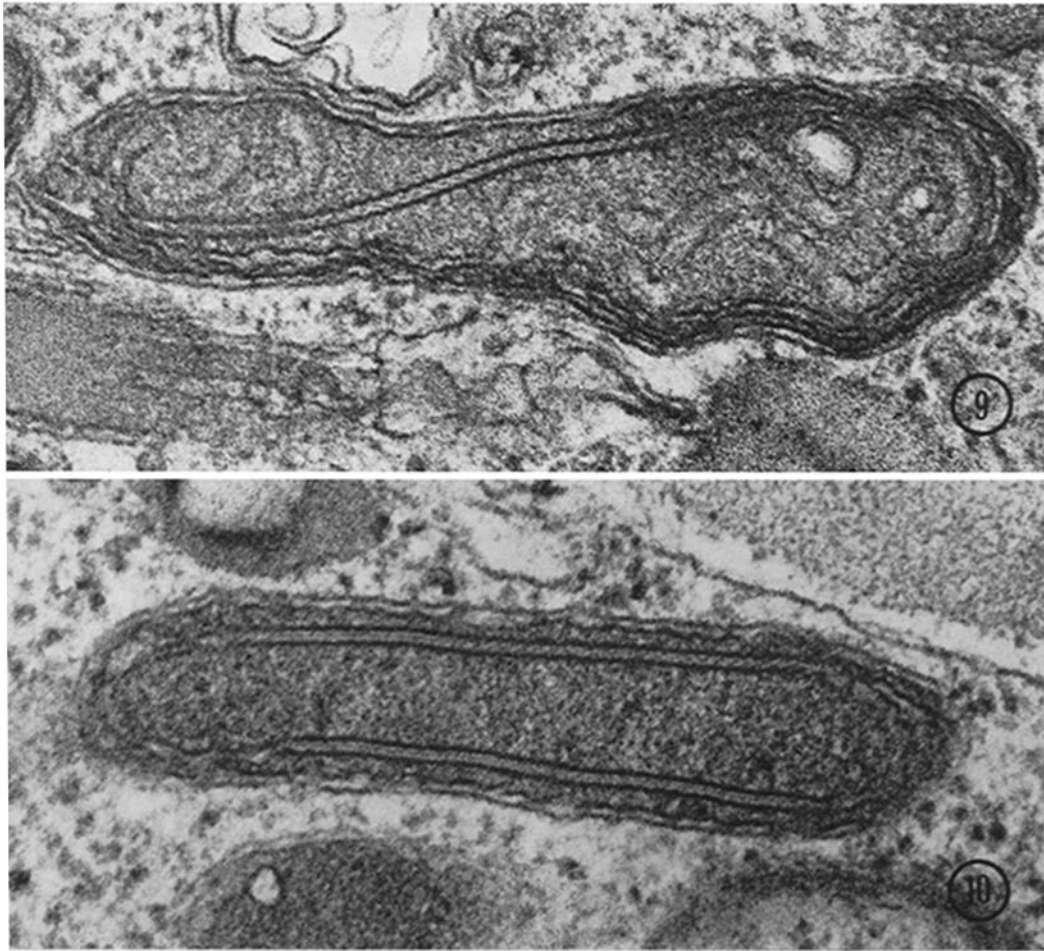
FIGURES 9 and 10 Mitochondria of wild type cells after 6 days of exposure to 200 μg/ml chloramphenicol. Fig. 9· The pentalaminar structure of the plate and its periodic appearance are shown. × 120,000. Fig. 10. Mitochondrion with two periodic plates. × 100,000.

as already suggested and discussed by several authors (for a comprehensive and detailed formulation, see Mahler et al , 1971). First, most mitochondrial enzymes are synthesized in the cytoplasm (for review, see Rabinowitz and Swift, 1970, and Mahler et al , 1971). Second, there is increasing evidence that the mitochondrial protein-synthesizing system contributes to the synthesis of some protein component(s) of the mitochondrial membrane (Roodyn, 1962, Wheeldon and Lehninger, 1966, Yang and Criddle, 1969; Tzagoloff, 1971) In vivo and in vitro studies of the protein-synthesizing activities of mitochondria from different organisms in the presence of radioactive amino acids have shown that the radioactivity is recovered in

an insoluble inner membrane fraction (Neupert et al , 1967, Beattie et al , 1967, 1970; Sebald et al , 1971; Neupert and Ludwig, 1971; Weiss et al , 1971)

At any rate, it must be stressed that both membranes of the mitochondrial envelope persist whereas the cristae are rapidly and drastically affected. An analogous result has been obtained by Linnane and coworkers on the yeast *Candida parapsilopsis* (Kellerman et al , 1969) and by Lenk and Penman on HeLa cells (1971). Thus, either the cristae alone contain elements which are sensitive to the blockage of mitochondrial protein synthesis or the cristae and the inner membrane have the same basic constitution but the turnover of the

cristal constituents is faster than that of the inner membrane and thus more sensitive to the blockage.

Aside from the disappearance of cristae, several important modifications of mitochondrial structure have been described (membrane pairs, plates) A possible filiation may exist among these modifications but the mechanism by which they arise and the extent of their possible dependence on blockage of mitochondrial protein synthesis are not known. It should be noted that rigid plates, resembling those described above, have been observed in several other different untreated cells: bean root (Newcomb et al , 1968), human glioblastoma (Tani et al , 1971), guinea pig liver (Valdivia, as reported by Green et al , 1971), beef heart (Hall and Crane, 1971). These plates, then, may not constitute a specific response to the antibiotic but may correspond to one among a limited number of possible configurations of mitochondrial structure under "unfavorable" conditions.

Aside from effects directly resulting from the inhibition of mitochondrial protein synthesis, indirect effects must also be considered. They may be related to the quite abnormal physiological state of the cells, or to some direct effect of the antibiotics on the respiratory chain (Beattie, 1968; Dixon et al , 1971), resulting in structural modifications (Harris et al , 1969). Particularly relevant may be the wavy cristae which resemble some configurations of energized mitochondria *in situ*, described by Harris et al. (1969). Also relevant are the unusual complex patterns described by Pappas and Brandt (1959), resulting from branching and fusing of "wavy" cristae in mitochondria of an *Amoeba*, and later shown by Daniels and Breyer (1968) to be related to starvation conditions

Nevertheless, the primary effect of antibiotics on mitochondrial protein synthesis is very likely to be the most important, and it is from this point of view that the comparison between sensitive and mutant cells is particularly significant Whereas antibiotics exert profound effects on the mitochondria of sensitive cells, they have little or no effect on resistant strains which not only can multiply but also can maintain normal mitochondria. The simplest interpretation is that the mitochondrial protein-synthesizing system itself is rendered resistant by the mutation, for instance, by a modification of a mitoribosomal protein as suggested by the results of Beale et al. (1972). This interpretation fits with the genetic data showing that the mutations are cytoplasmically inherited and are most likely located in the mitochondrial DNA

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