

## EFFECTS OF ETHIDIUM BROMIDE ON THE CYTOCHROME CONTENT AND ULTRASTRUCTURE OF L CELL MITOCHONDRIA

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### ABSTRACT

Ethidium bromide intercalates between the bases of native DNA, resulting in several biological anomalies. The effects of ethidium bromide on the mitochondria of cultured mouse L cells were studied. At a concentration of  $1 \mu\text{g}$  ethidium bromide/ml it was observed that concentrations of cytochromes  $a + a_3$  and  $b$  decreased,  $a + a_3$  more rapidly than  $b$ . In contrast, the concentration of cytochromes  $c_1$  and  $c$  increased or remained the same as in control cells. Concomitant with the decrease of cytochromes  $a + a_3$  and  $b$  was an enlargement of the mitochondria and a reduction in the cristae. The cristae that remained were abnormally organized. After prolonged treatment with ethidium bromide a second population of small, more normally organized mitochondria was apparent. These effects of ethidium bromide could be reversed.

### INTRODUCTION

The list of effects elicited by ethidium bromide (EB) in living organisms has been growing since the early 1960's. The phenanthridine dye, ethidium bromide, intercalates between the bases of double-stranded DNA (1, 2). This effect potentially leads to interference with the replication and transcription of the genome. Several adverse effects of EB on the synthesis and structure of mitochondrial DNA (3, 4) and kinetoplast DNA (5) have since been described.

Other effects of EB include the preferential inhibition of mitochondrial DNA polymerase in vitro as compared with the nuclear enzyme using the same DNA template (6). During EB treatment of L cells in vivo, thymidine- $^3\text{H}$  continues to be incorporated into nuclear DNA at an apparently greater rate than that in untreated cells (4). EB has also been shown to inhibit the synthesis of RNA present in mitochondria (7, 8).

Ethidium bromide induces cytoplasmic mutations in some cell types. In trypanosomids, EB treatment leads to loss of the kinetoplast (9). Slonimski and co-workers (10) have demonstrated that EB is an extremely efficient cytoplasmic mutagen in facultative anaerobic yeast. The "petites" obtained after treatment with EB lack cytochromes  $a + a_3$  and  $b$ . In obligate aerobic yeast, however, the intercalating agents euflavine and EB inhibit the synthesis of  $a + a_3$ ,  $b$ , and  $c_1$  but do not induce the petite mutation (11).

The studies described here extend the effects of EB to mammalian cells. De Vries and Kroon (12) recently showed that EB inhibits the synthesis of cytochromes  $a + a_3$  in regenerating rat liver. These workers could not reverse the effects of EB in cultured heart cells. Our studies with cultured L cells demonstrate the reduction or loss of cytochromes  $a + a_3$  and  $b$ , with a concomitant

increase in the concentration of cytochrome *c* during 2–3 days of EB treatment. Mitochondrial profiles become greatly enlarged, with few abnormally organized cristae remaining. The effects were found to be reversible and similar to those described for obligate aerobic yeast treated with intercalating dyes (11).

## METHODS AND MATERIALS

### *Cell Culture Procedures*

L cells were cultured in Joklik medium (Grand Island Biological Co., Grand Island, N. Y.) in spinner flasks and were maintained at 37°C. Ethidium bromide was added from a freshly prepared stock solution of 200 µg/ml under sterile conditions.

### *Mitochondria*

The number of L cells employed for each spectroscopic determination ranged from  $2 \times 10^8$  to  $1 \times 10^9$  cells. All manipulations were carried out at 4°C. Cells were harvested by centrifugation at 235 *g* for 10 min in a Sorvall centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) The cells were resuspended in  $1 \times$  phosphate-buffered saline (PBS) (8 g NaCl; 0.2 g KCl; 1.15 g Na<sub>2</sub>HPO<sub>4</sub>; 0.2 g K H<sub>2</sub>PO<sub>4</sub> in 1 liter) and centrifuged at 175 *g* for 10 min. The cell pellet was suspended in 20 ml of cold 0.05% bovine serum albumin (in 25 mM Tris buffer, pH 7.4, 2 mM ethylenediaminetetraacetate [EDTA]) and homogenized in a Dounce homogenizer (Kontes Glass Co., Vineland, N. J.) with 20 passes using a tight plunger. 20 ml of 0.6 M sucrose (in Tris-EDTA) was added and the solution was homogenized with three more passes. The homogenate was centrifuged two times in a Sorvall at 750 *g* for 10 min, each time discarding the nuclear plus cell debris pellet. The supernate was then centrifuged at 10,900 *g* for 20 min, yielding a crude mitochondrial pellet which was usually suspended in 1 ml of 0.3 M sucrose and frozen for subsequent spectral analysis. Mitochondria were not purified further so as to obtain a random sample of the total population and, more important, to keep the loss of cytochrome *c* at a minimum. It has been assumed that the purity of the mitochondrial sample is fairly constant between preparations.

### *Spectroscopic Analysis of Mitochondrial Cytochromes*

The method of Williams (13) for the simultaneous quantitative estimation of cytochromes *a*, *b*, *c*<sub>1</sub> and *c* in mitochondria was used. The split beam spectrophotometer of Chance (14) was employed to obtain the spectra. Base line corrections were made using the

isobestic points at 530, 580, and 615 mµ. The points at 530 and 615 mµ were taken from work by Chance and Schoener (14). The 580 mµ point is an approximation based on the fact that 577 mµ is the valley point for cytochrome *b* (lies below 0 OD in the difference spectrum) and that the absorption at 580 mµ due to cytochromes *a* and *c* is negligible (15, 16).

### *Electron Microscopy*

10 ml samples of L cells grown in suspension culture were added to 1 ml of 2% OsO<sub>4</sub>–0.1 M phosphate buffer (pH 7.4) at 0°C for 10 min. The cells were centrifuged and resuspended in 2% OsO<sub>4</sub>–0.1 M phosphate buffer (pH 7.4) at 0°C for 1 hr. The cells were washed in phosphate buffer and postfixed with freshly prepared 10% paraformaldehyde in the same buffer for 20 hr at 4°C. The specimens were washed in buffer, dehydrated in a graded series of ethyl alcohol, and embedded in Araldite. The sections were stained with uranyl acetate followed by lead citrate and examined in a Siemens Elmiskop Ia electron microscope.

## RESULTS

### *Spectroscopic Studies*

The results shown in Tables I and III are representative of the effects of EB on L cells. There is some variation in the growth patterns of L cells from day to day and from one culture to another. There is also a variation in the extent of the response to EB with different cultures. However, in all of the experiments that were conducted, the general effect of EB on the L cells was the same. Cell growth is diminished. For the experiment shown in Table I, control cells doubled in number every 24 hr whereas EB-treated cells grew about 80% after the first day, 6% after the second day, and 15% after the third day. The concentration of cytochrome *a* decreases more rapidly than the concentration of cytochrome *b*. In a separate experiment (results not shown here), difference spectra were obtained using rotenone (oxidized) versus sodium dithionite (reduced) which permits the recording of the Soret region. Spectra were obtained with mitochondria from L cells treated with EB (1 µg/ml) for 24, 48, and 72 hr and were compared to the spectra of mitochondria from untreated cells. The absorption difference between 445 and 460 mµ (absorption region of *a*<sub>3</sub> + *a*) showed a decrease comparable to the changes in the visible region for cytochrome *a*. In marked contrast to cytochromes *a* and *b*, the con-

TABLE I  
Changes in Cytochrome Concentration during Treatment of L Cells with Ethidium Bromide\*

Sample	μmoles cytochrome/mg mitochondrial protein					Ratio (a:b:c <sub>1</sub> + c)
	a	b	c <sub>1</sub>	c	c <sub>1</sub> + c	
Control	0.122	0.128	0.066	0.086	0.152	1:1.05:1.24
1 μg/ml EB (24 hr)	0.102	0.163	0.085	0.097	0.152	1:1.60:1.78
1 μg/ml EB (48 hr)	0.065	0.118	0.076	0.131	0.207	1:1.82:3.18
1 μg/ml EB (72 hr)	0.046	0.074	0.062	0.095	0.157	1:1.61:3.41
2 μg/ml EB (24 hr)	0.095	0.133	0.077	0.125	0.202	1:1.40:2.13
2 μg/ml EB (48 hr)	0.071	0.096	0.069	0.107	0.176	1:1.35:2.48
2 μg/ml EB (72 hr)	0.066	0.117	0.076	0.125	0.201	1:1.77:3.04

\* Spectra taken at room temperature.

TABLE II  
Changes in Cytochrome Ratios during Treatment of L Cells with Ethidium Bromide

Sample	Ratios		
	a = 1 (a:b:c <sub>1</sub> :c)	b = 1 (b:c <sub>1</sub> :c)	c <sub>1</sub> = 1 (c <sub>1</sub> :c)
* Control	1:1.99:2.03:2.23	1:1.02:1.12	1:1.10
* 1 μg/ml EB (24 hr)	1:2.03:2.06:2.49	1:1.02:1.23	1:1.21
* 1 μg/ml EB (48 hr)	1:3.61:4.03:4.18	1:1.12:1.16	1:1.04
* 1 μg/ml EB (96 hr)	1:4.37:5.32:8.99	1:1.22:2.06	1:1.69
† Control	1:1.05:0.54:0.70	1:0.52:0.67	1:1.30
† 1 μg/ml EB (24 hr)	1:1.60:0.83:0.95	1:0.52:0.60	1:1.14
† 1 μg/ml EB (48 hr)	1:1.82:1.17:2.02	1:0.64:1.11	1:1.72
† 1 μg/ml EB (72 hr)	1:1.61:1.35:2.07	1:0.84:1.28	1:1.53

\* Spectra taken at liquid nitrogen temperature. Ratios are from absorption peaks for individual cytochromes.

† Ratios are from results at room temperature in Table I and are determined from concentrations of the individual cytochromes.

centration of cytochromes  $c + c_1$  increases after the addition of EB to the culture medium. This effect is the same at concentrations of EB ranging from 1 to 5 μg EB/ml. However, 5 μg EB/ml is a lethal dose when cells are subjected to this concentration for more than 2 days (there is some variation).

The effect of EB on the concentration of cytochrome  $c$  is difficult to determine from room temperature spectra. In order to check the room temperature results, an experiment, similar to Table I, was conducted and spectra were taken at the temperature of liquid nitrogen. This permits the resolution of the  $c_1$ ,  $c$ , and  $b$  peaks. Ratios of the mitochondrial cytochromes were determined and compared qualitatively to the ratios obtained from the room temperature data. Good

qualitative agreement between the low temperature and room temperature experiments is shown in Table II.

After 120 hr of exposure to 1 μg EB/ml the L cells have virtually ceased growing. At this point, as can be seen in Table III, the concentrations of cytochromes  $a$  and  $b$  are extremely low while the concentration of cytochrome  $c + c_1$  is only slightly depressed. The decreased cytochrome  $a + a_3$  and  $b$  concentrations cannot be accounted for by greater amounts of mitochondrial protein per cell in EB-treated preparations. (This increase over controls does not exceed 30%. It is also found even after the mitochondria are purified on a sucrose gradient.) The reduction of the cytochrome  $c$  level, relative to control values after 5 days of EB treatment, probably reflects an over-all reduction

TABLE III  
Changes in Cytochrome Concentration during Recovery of *L* Cells from Treatment with Ethidium Bromide\*

Sample	μmoles cytochrome/mg mitochondrial protein					Ratio (a:b:c <sub>1</sub> + c)
	a	b	c <sub>1</sub>	c	c <sub>1</sub> + c	
Control	0.087	0.114	0.059	0.072	0.131	1:1.31:1.51
1 μg EB (120 hr)	0.014	0.011	0.036	0.048	0.084	1:0.79:6.00
48 hr reversal	0.016	0.007	0.014	0.073	0.087	1:0.44:5.44
96 hr reversal	0.037	0.039	0.036	0.105	0.141	1:1.05:3.81
168 hr reversal	0.060	0.082	0.041	0.091	0.131	1:1.37:2.20
216 hr reversal	0.101	0.119	0.062	0.101	0.163	1:1.18:1.61

\* Spectra taken at room temperature.

of protein synthesis. These *L* cells, after 120 hr of EB treatment, were centrifuged, washed, and then resuspended in fresh Joklik medium without EB. The cells were capable of reversing their growth rate, and by 168 hr were growing at normal rates (approximately doubled in 24 hr). Concomitant with the reversal of their growth pattern was a return to a normal cytochrome composition, as seen in Table III.

#### Ultrastructural Studies

Fig. 1 shows the ultrastructure of a normal *L* cell grown logarithmically in suspension culture. The mitochondria of control cells are filamentous, ranging from short rods to longer, sometimes branched filaments. The mitochondrial profiles are filled with well-defined cristae, except for some clear regions in the matrix in which DNA is usually located (17).

Cells treated with either 1 μg or 2 μg of ethidium bromide per ml over a period of 7 days show a series of striking changes in the mitochondrial ultrastructure (Figs. 2-9). There is some variation in different experiments as to the extent of these changes and the time at which the changes can be observed. The following represents a typical experiment. During the first 2 days of treatment, most of the mitochondrial profiles become enlarged and the cristae are reduced in number and size (4). A few mitochondrial profiles remain apparently unchanged. After 3 and 4 days of EB treatment, giant mitochondrial profiles are abundant (Figs. 2-4). The matrix is less dense than in control cells. There is a diversity of internal structure in these mitochondria within a given cell. Some profiles completely lack cristae, and others have very short cristae along the periphery (Figs. 2, 4). Some mitochondrial cristae traverse the

entire width of the mitochondrion, possibly separating it into distinct compartments (Figs. 2, 4). A honeycomb pattern of cristae is apparent in some mitochondria, and cross-sections of tubular cristae consisting of two concentric rings are also common (Fig. 3). The matrix of some mitochondria contains a very prominent electron-opaque fibrous material (Figs. 2, 4). It is not known how or whether this material is related to DNA, as its ultrastructural appearance is more granular than that of typical mitochondrial DNA (17). Concentrically arranged groups of vesicles are frequently located in the cytoplasm near mitochondria.

Between 5 and 7 days of treatment with EB, two general trends are apparent in the mitochondrial population. First, mitochondria begin to appear more dense than before. Giant mitochondria are still apparent, but many of them tend to have more cristae than before which are frequently arranged in circular patterns within the mitochondrial profile (Figs. 5, 6). Second, a set of small mitochondria becomes apparent whose ultrastructure suggests that they originate from portions of the large mitochondria (Fig. 5). Both the outer and inner membrane are apparent even in the mitochondrial profiles that are almost devoid of cristae (Fig. 7). The proportion of small dense mitochondria is increased after 7 days of EB treatment (Fig. 8). Whether the small dense types are more resistant to the effects of EB than the large empty types can only be speculated upon.

The morphological effects after removal of ethidium bromide showed a gradual return to almost normal mitochondrial ultrastructure. After a 3-day or 6-day treatment with EB, followed by 2 and 3 days of growth in normal medium, two populations of mitochondria were still apparent:

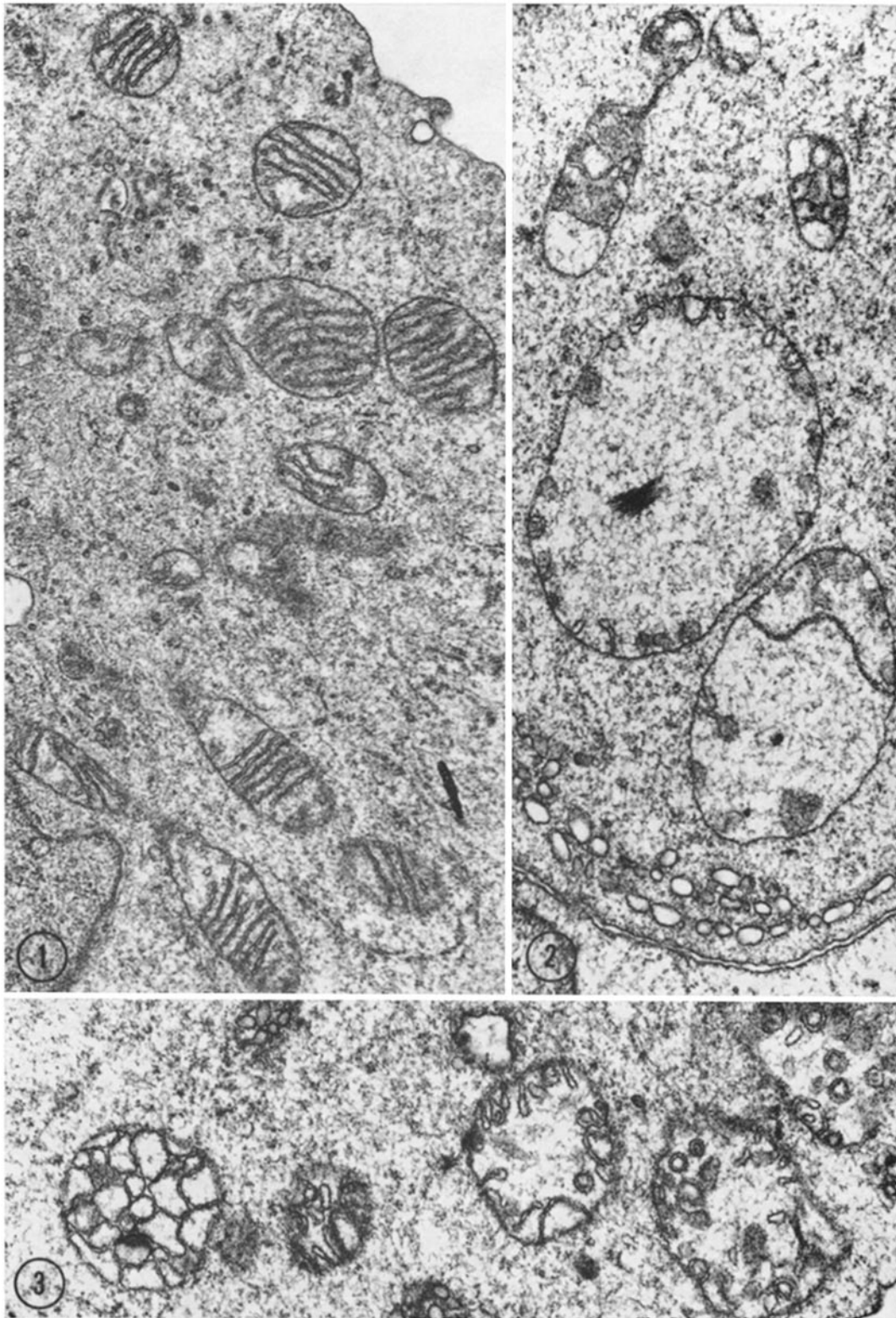


FIGURE 1 Electron micrograph of a control L cell, showing several typical profiles of mitochondria.  $\times 24,000$ .

FIGURE 2 L cell grown in the presence of 1  $\mu\text{g/ml}$  ethidium bromide (EB) for 3 days. Mitochondria are greatly enlarged and the amount of cristae appears reduced. Lower mitochondrion is transected by a crista.  $\times 24,000$ .

FIGURE 3 Changes in the structure of some of the L cell mitochondria after exposure of cells to EB (2  $\mu\text{g/ml}$ ) for 3 days. Mitochondrial profiles show scallop-shaped and tubular cristae, some of which appear to form separate circular subunits.  $\times 24,000$ .

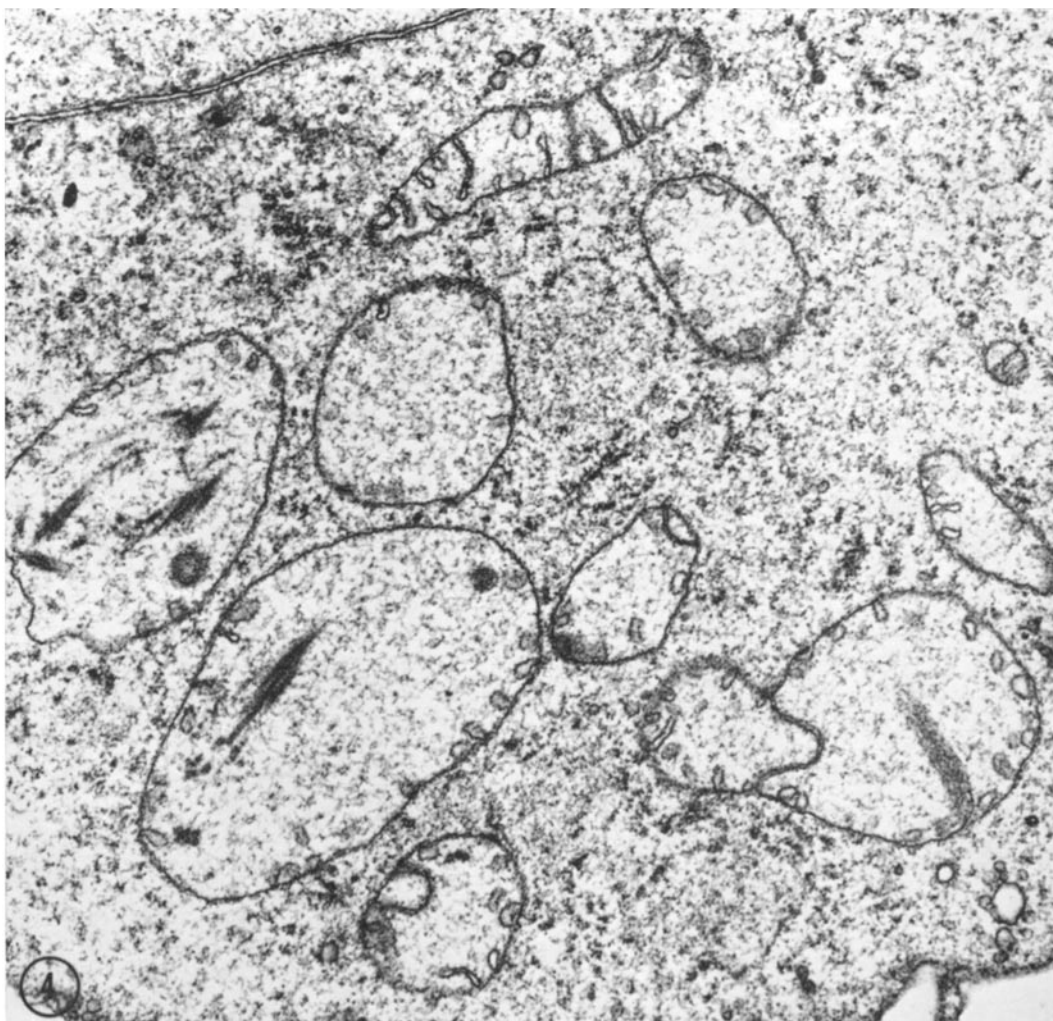


FIGURE 4 Greatly enlarged mitochondria in cells grown in the presence of EB for 4 days. Mitochondrion in lower right appears divided into two compartments. Mitochondrion at top appears almost normal.  $\times 24,000$ .

small dense types and the large swollen types (Figs. 9, 10). The latter type had more cristae than before reversal, and these cristae appeared frequently disorganized. Large numbers of concentrically arranged membranes of the endoplasmic reticulum were common in the cytoplasm. After 7 days in normal medium, about equal numbers of small dense mitochondria and larger, almost normal ones were encountered. The latter type predominated after 15 days of reversal (Fig. 11).

#### DISCUSSION

Ethidium bromide appears to be an excellent tool for studying mitochondrial biogenesis. All studies

to date (see Introduction) indicate that at low concentrations the most pronounced known effects of the action of ethidium bromide are found in the mitochondrion. Therefore, at least some of the changes elicited by EB in the mitochondrion may be tacitly assigned to a mitochondrial rather than a nuclear control mechanism. The action of EB may be direct or indirect. The precise mechanism of action of this drug in vivo, however, remains to be determined.

The results of several experiments (one typical experiment shown in Table I) demonstrate a rapid decrease in the amounts of cytochromes *a* and *b* at concentrations of EB ranging from 1 to 5  $\mu\text{g/ml}$ .



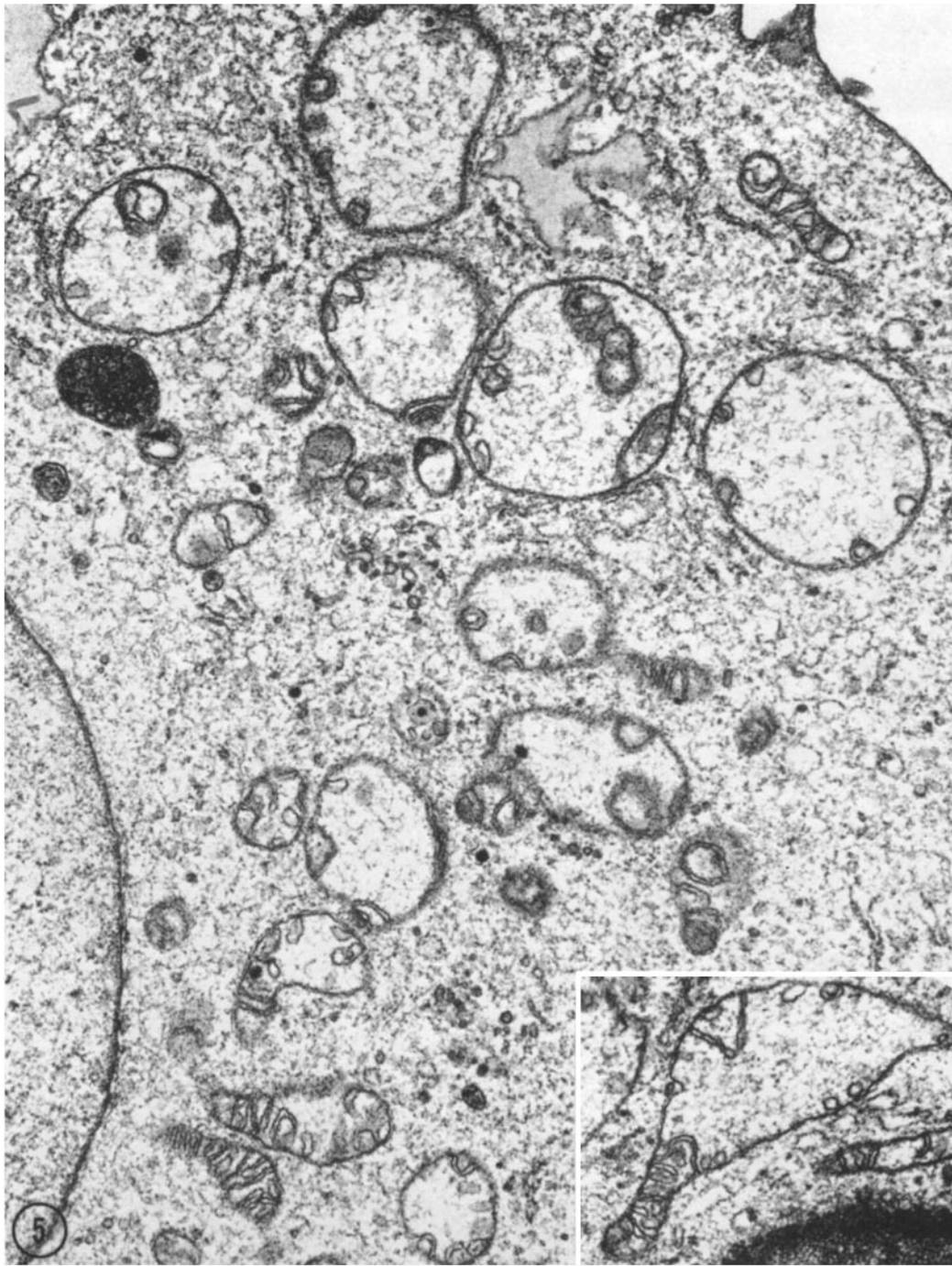


FIGURE 5 L cell grown in the presence of 2  $\mu\text{g}/\text{ml}$  EB for 6 days. Mitochondrial profiles show regions with whorllike or tubular cristae. Some cristae are organized in one narrow portion of an otherwise enlarged mitochondrion (inset). Some small mitochondrial profiles (upper right and lower left) are similarly organized as the localized regions with cristae in giant mitochondria.  $\times 24,000$ .

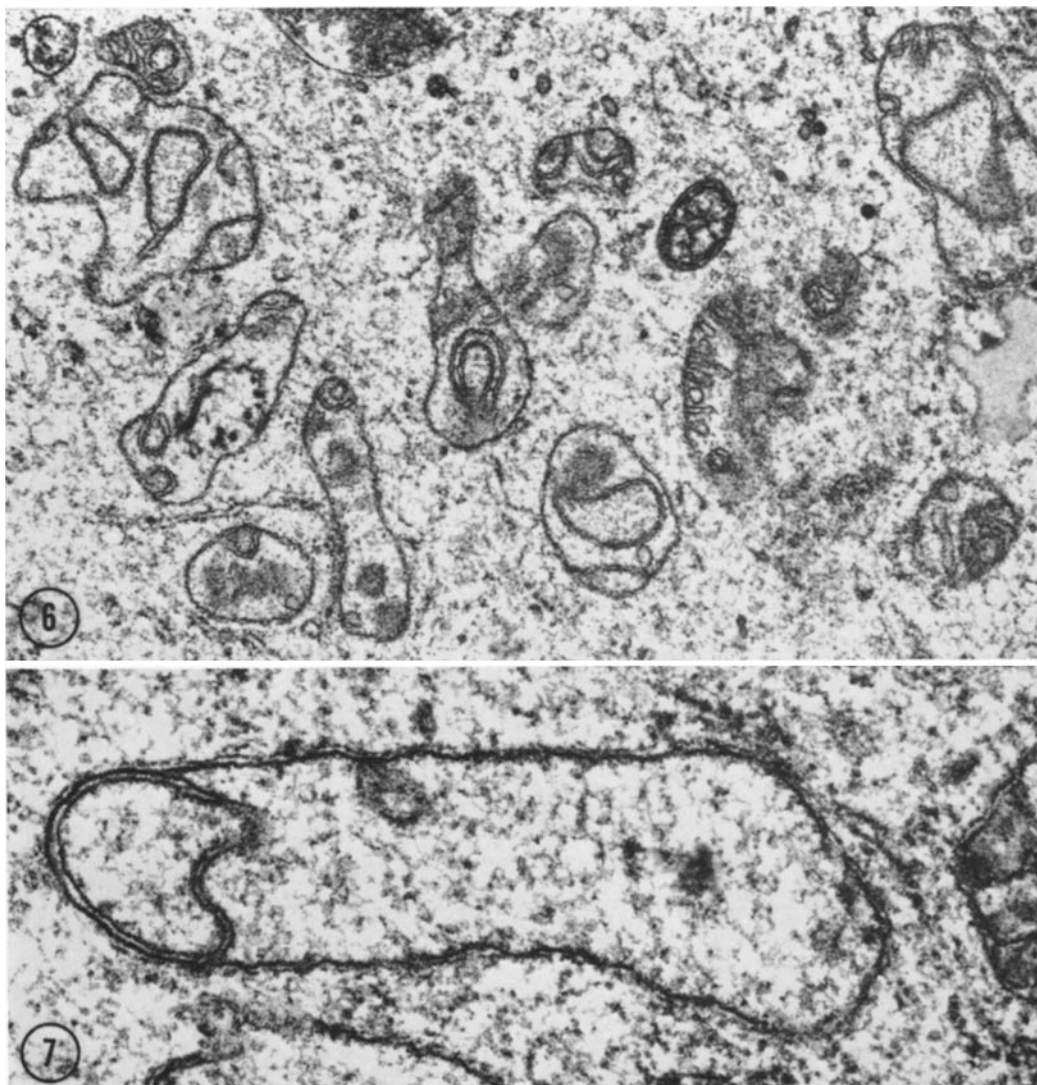


FIGURE 6 Part of cell from the same material as in Fig. 5, illustrating circularly arranged cristae.  $\times 24,000$ .

FIGURE 7 Mitochondrial profile from the same material as in Fig. 5, showing the presence of the outer double membrane and partitioning of the organelle.  $\times 60,000$ .

Cytochrome *a* is calculated by the difference in the peak to valley absorption at 605 and 630  $m\mu$ . The contribution by cytochrome *a*<sub>3</sub> to the absorption in this region is approximately 20% (18). It is, therefore, difficult to conclude that the concentration of cytochrome *a*<sub>3</sub> declines along with that of cytochrome *a*. However, in the experiment where difference spectra were obtained using rotenone

(oxidized) versus sodium dithionite (reduced), a comparison of the absorption difference between 445 and 460  $m\mu$  of mitochondria from control cells and mitochondria from cells treated with EB (for 24, 48, and 72 hr) showed a decrease comparable to the changes in the visible region. This indicates that cytochrome *a* + *a*<sub>3</sub> is affected by EB as a whole. The drop in the cytochrome *b* con-



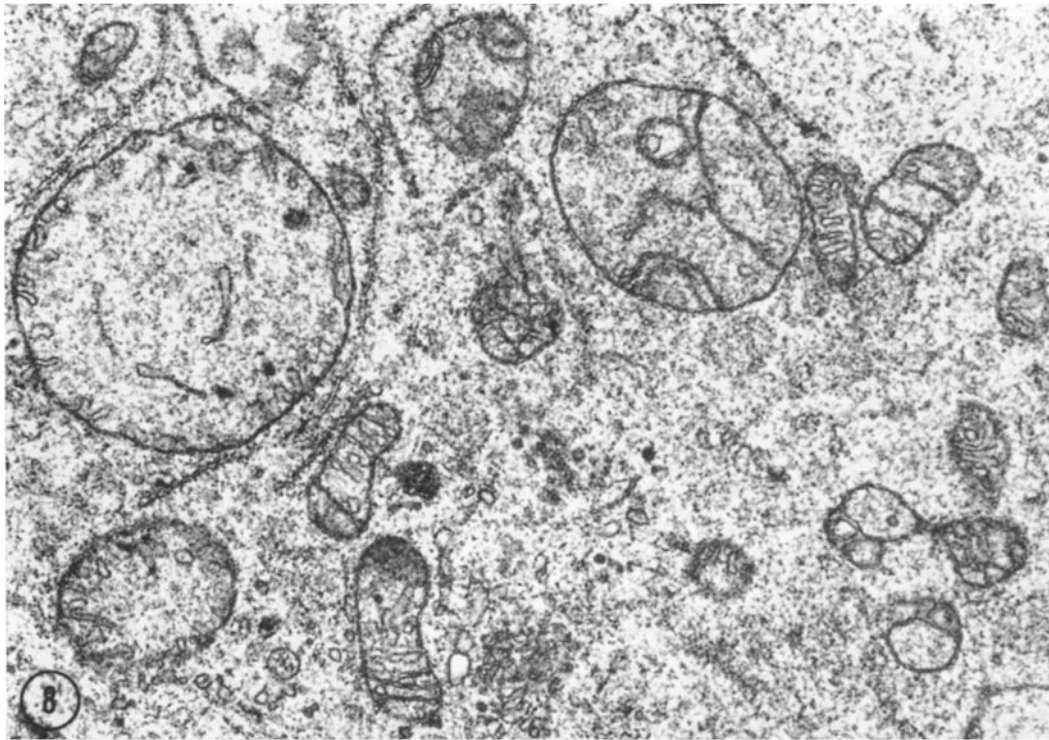


FIGURE 8 L cell grown in the presence of 2 µg/ml EB for 7 days. Both enlarged and small cristae-filled mitochondrial profiles are present.  $\times 24,000$ .

centration is not as rapid as that of cytochrome  $a + a_3$  but after 5 days of EB treatment (Table III) both are at approximately the same level.

Cytochrome  $c + c_1$  behaves in an opposite fashion with respect to cytochromes  $a + a_3$  and  $b$ . The concentration of cytochrome  $c$  increases with EB treatment and begins to fall off only after about 5 days of exposure to EB. The eventual decline of cytochrome  $c$  at this late stage of EB treatment can probably be attributed to cellular impairment of the protein synthetic mechanism preceding cell death. Vital dye tests show that virtually all of the cells are alive at this point. After 7 days of treatment, 45–65% of the cells are still alive. Cytochrome  $c_1$  appears to behave like cytochrome  $c$ . In agreement with the room temperature data, the results of low temperature spectroscopy (Table II) show that the ratio of  $c_1$  to  $c$  remains constant after at least 2 days of EB treatment. Both  $c_1$  and  $c$  increase with respect to cytochrome  $c_1$  after several days of exposure to EB. This evidence strongly indicates that  $c_1$  follows the same pattern as cyto-

chrome  $c$  and does not decrease, as is the case of  $a + a_3$  and  $b$ .

The electron micrographs support the spectroscopic findings. The reduction or loss of cytochromes  $a + a_3$  and  $b$  may be correlated with a striking reduction of the amount of mitochondrial cristae. The remaining part of the inner mitochondrial membrane which follows the outline of the outer membrane in the enlarged mitochondria may be an entity that differs in some respects from the cristae, or the cristae may have completely or partially unfolded into the peripheral inner membrane (11). Whatever reorganization of inner membranes has taken place, the total amount of these membrane and “insoluble” cytochromes appears less in EB-treated than in control cells. The small dense mitochondria appearing after prolonged treatment with EB may be resistant forms and probably account, at least partially, for the remaining low levels of cytochromes  $a + a_3$  and  $b$ . Whether some of the small mitochondria are derivatives of the large types, as it appears in some electron micro-

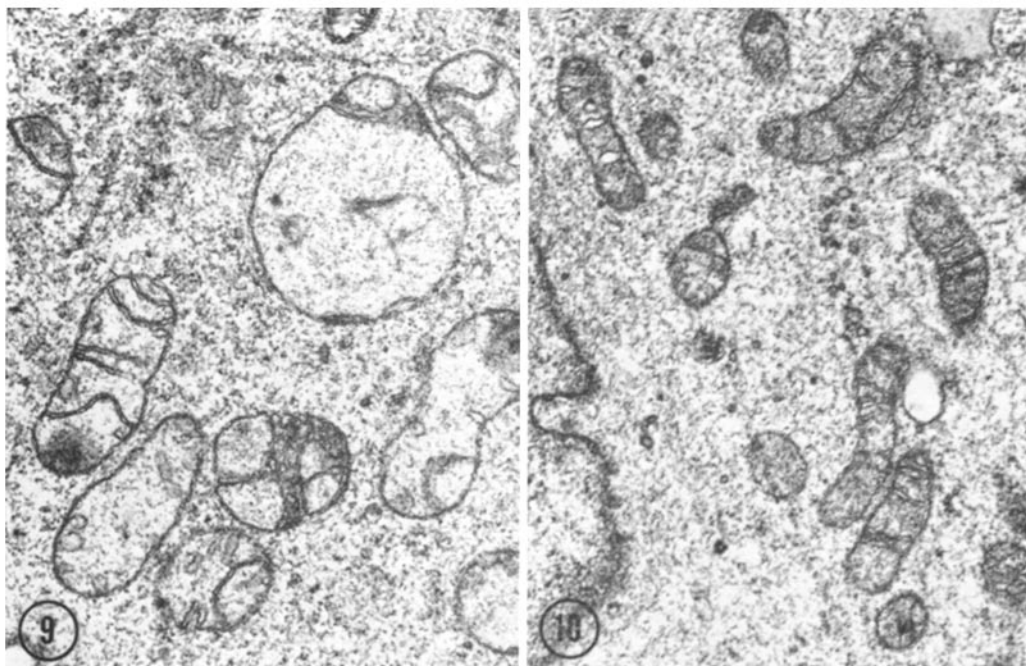


FIGURE 9 L-cell grown in normal medium for 2 days after exposure to EB ( $2\text{ }\mu\text{g/ml}$ ) for 3 days. Mitochondria of most cells are smaller and more cristae are visible than during EB treatment.  $\times 24,000$ .

FIGURE 10 L cell grown in normal medium for 2 days after exposure to EB ( $2\text{ }\mu\text{g/ml}$ ) for 3 days. In addition to cells as in Fig. 9, many cells contain small dense mitochondria.  $\times 24,000$ .

FIGURE 11 L cell grown in normal medium for 15 days after exposure to EB ( $2\text{ }\mu\text{g/ml}$ ) for 5 days. Mitochondria are filled with cristae and appear similar to those in control cells (Fig. 1), although some mitochondria are larger.  $\times 24,000$ .



graphs, remains to be determined. Separation and analysis of the different populations of mitochondria may give further clues to this problem.

Two important conclusions can be drawn from these results. First, the fact that the cytochrome *c* concentration increases demonstrates a relatively intact mitochondrial membrane after EB treatment. Morphologically intact double membranes can also be seen in the electron micrographs of EB-treated cells even after prolonged treatment. Secondly, EB treatment is known to impair selec-

tively M-DNA synthesis and structure (4) and M-protein synthesis (19). The present results, concomitant with these findings, support the theory that mitochondria control at least in part the synthesis of cytochromes *a* + *a*<sub>3</sub> and *b* (regardless of the source of messenger RNA), while cytochrome *c* (*c*<sub>1</sub>) is coded for by the nucleus and synthesized in the cytoplasm.

The effects of EB can be reversed by resuspending the L cells in fresh medium lacking EB, as can be seen in Table III. Reversibility is also demon-

strated in the electron micrographs. The present studies do not indicate what mechanism is involved in the reversal of EB-treated cells.

It is difficult to draw any definite conclusions about the mutagenic properties of EB in the mammalian L cells. The multiple populations of "abnormal" and "normal" mitochondria, seen in the electron micrographs, in EB-treated cells might indicate some degree of autonomy for mitochondria. A fraction of the mitochondria within a single cell may be more resistant to EB than others. Mutations may occur within a particular mitochondrion rather than a whole cell. Such a situation would account for the loss of some but not all of the cytochromes  $a + a_3$  and  $b$ . It would also explain reversibility since those mitochondria that are defective would not replicate whereas the resistant mitochondria would. Studies under progress that may show the fate of each type of mitochondrion might help to clear up these points. The experiments do not exclude the possibility that some cells as a whole are resistant to EB whereas others are not. The nonresistant cells may form mutants lacking cytochromes  $a + a_3$  and  $b$  which upon reversal are selectively lost since they are incapable of growing anaerobically. A third possibility exists where EB is not mutagenic in mammalian cells. In this case a different mechanism would have to be postulated for the action of EB than is found in lower forms, such as facultative anaerobic yeast.

The increase in cytochromes  $c + c_1$  may be an expression of the interaction of the nucleus and the mitochondrion. If only a portion of the mitochondria per cell are transformed by EB into respiratory-deficient mitochondria, the nucleus might interpret the situation as a loss of mitochondria and therefore direct the synthesis of mitochondrial proteins that fall under its control. This is supported by an increase of nuclear DNA synthesis and mitochondrial protein per cell upon EB treatment (4).

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