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The Reversibility of the Ethidium Bromide–Induced Alterations of Mitochondrial Structure and Function in the Cellular Slime Mold, *Dictyostelium discoideum*

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Abstract

Addition of ethidium bromide to ameboid cultures of the slime mold, *Dictyostelium discoideum*, caused a cessation of cell division after 1 or 2 generations. The replication of mitochondrial DNA was immediately blocked as indicated by the 50% decrease in the DNA content of purified mitochondria from ethidium-bromide-treated cultures. The activity of the respiratory chain was also inhibited, resulting in a 75% decrease in cyanide-sensitive whole cell respiration. Spectral analysis at low temperature indicated that the amount of cytochrome c_1 was decreased 80% and that of cytochrome *c* increased 100% in mitochondria from treated cells. Two cytochromes *b* absorbing at 556 and 561 nm were observed in mitochondria from both control and ethidium-bromide-treated cultures. The content of cytochrome b_{561} appeared to decline more than did b_{556}, but it is hard to quantitate the decrease. The effects of ethidium bromide were fully reversible. When the drug was removed, the cells resumed a normal growth rate without any discernible lag. The activity of oligomycin-sensitive ATPase, cytochrome oxidase, and succinate–cytochrome-*c* reductase as well as the cytochrome content began to increase after 1 day returning to control levels within 5 days. Electron micrographs of whole cells

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treated with ethidium bromide revealed that mitochondrial profiles were elongated and had greatly reduced cristae. Numerous membrane whorls were apparent, as was a profound loss of rough endoplasmic reticulum. Three days after removal of ethidium bromide, mitochondria were again ovoid in shape and contained well-developed cristae. In all of the cells during recovery, there was a single large vacuole that appeared to enclose a large portion of the cell volume, forming a new cellular compartment that may simplify the breakdown of previously damaged organelles.

Introduction

Ethidium bromide and the acridine dyes that preferentially intercalate between the bases of covalently closed circular DNA [1] have been shown to be specific inhibitors of the replication and transcription of mitochondrial DNA [2, 3]. Exposure of facultatively aerobic yeast to ethidium bromide results in the irreversible formation of cytoplasmic petites characterized by a complete loss of all mitochondrial cytochromes except cytochrome *c* [4]. By contrast, ethidium bromide treatment of various other cell types, such as human fibroblasts [5], mouse L cells [6, 7], SV-40 transformed cells [8], *Tetrahymena* [9], or *Euglena* [10], results in alterations in mitochondria that are completely reversible following removal of the drug.

The cellular slime mold, *Dictyostelium discoideum*, is an excellent organism for studies of mitochondrial biogenesis as well as the role of mitochondria during development. The slime mold grows logarithmically as a unicellular amoeba with a generation time of 12–15 hr when supplied with adequate nutrients. Under controlled conditions, the amoebae aggregate into a multicellular mass and synchronously differentiate into two distinct cell types. Previously, we reported that after addition of ethidium bromide to cultures of slime mold amoebae, cell division ceased after one or two generations [11]. Purified mitochondria obtained from cultures treated with ethidium bromide for 5 days, a time equivalent to 8–10 generations, had an approximately 80% decrease in activity of both cytochrome oxidase and oligomycin-sensitive ATPase and a 50% decrease in succinate–cytochrome-*c* reductase activity [12]. By contrast, no change in the activity of succinate and NADH dehydrogenases was observed in mitochondria from either control or ethidium-bromide-treated cells; however, the amount of mitochondrial protein per cell increased significantly.

In the present study the characterization of mitochondria obtained from the slime mold after treatment with ethidium bromide has been extended both biochemically and morphologically. The complete reversibility of

these effects has been demonstrated. In addition, the kinetics of the recovery of respiratory chain components has been investigated.

Materials and Methods

Cell Culture and Preparation of mitochondria. Axenic cultures of *Dictyostelium discoideum*, strain A–3, were grown at 22–23°C as described previously [11]. When the cells had grown to a density of $0.5–2.0 \times 10^6$ cells per ml, ethidium bromide was added to a final concentration of 10 μg per ml. The flasks were wrapped with aluminium foil to prevent breakdown of light-sensitive ethidium bromide and incubated on a New Brunswick gyratory shaker at 200 rpm. Cells used for controls were grown to a density of $1–3 \times 10^6$ cells per ml. Stationary phase is reached at a density of $1–1.5 \times 10^7$ cells per ml. Mitochondria were prepared from control and ethidium-bromide-treated cells by sucrose gradient centrifugation as described previously [12].

Enzyme Assays. Cytochrome *c* oxidase, succinate–cytochrome-*c* reductase, succinate dehydrogenase, NADH dehydrogenase, and NADH–cytochrome-*c* reductase were each assayed at 23°C in a Gilford spectrophotometer as described by Kim and Beattie [13]. ATPase activity was measured at 22°C in a medium containing 3.0 mM $MgCl_2$, 10 mM ATP, 50 mM tris·HCl, pH 9.0, and approximately 1.0 mg of mitochondrial protein in a final volume of 1.0 ml [12]. Aliquots were removed at 1-min intervals over a 5-min period. The reaction was terminated by addition of 0.5 ml of 6.6% perchloric acid, and following centrifugation 1.0 ml of the clear supernatant was removed for phosphate analysis.

Oxygen uptake was determined at 20°C with a Clarke oxygen electrode. Approximately 1.0–1.5 mg mitochondrial protein or 3–4 mg whole cell protein was added to 3 ml of a buffer containing in 200 ml, 10.93 g mannitol, and 20 ml each of a 0.1 M solution of tris, pH 7.5, KCl, and KH_2PO_4. Succinate and ADP were added to a final concentration of 3.3 and 0.5 mM, respectively.

Chemical Determinations. Mitochondrial DNA was determined by the method of Morse and Carter [14]. Gradient purified mitochondria were precipitated in 10% cold trichloroacetic acid. Following centrifugation at 755 *g* for 20 min, the supernatant was discarded and the pellet was hydrolyzed in 2.0 ml of a 5% trichloroacetic acid solution by boiling for 30 min. The suspension was brought to 2.0 ml with 5% trichloroacetic acid, centrifuged at 755 *g* for 15 min, and the supernatant assayed for deoxyribose content according to the method of Sevag et al. [15] using calf thymus DNA type 1 (sodium salt) as standard. The assay consisted of

adding two volumes of diphenylamine reagent to 1 volume of unknown, heating in a boiling water bath for 10 min and recording the absorbance at 595 nm on a Gilford spectrophotometer. Diphenylamine was recrystallized from 70% ethanol and made 1% w/v in glacial acetic acid. Concentrated H_2SO_4 (2.75 ml) was added to 100 ml of this acid solution. Protein was determined by the method of Lowry et al. [16] using bovine serum albumin as standard.

Spectral Studies. The room-temperature difference spectrum of whole cells or purified mitochondria was determined in a Cary model 15 spectrophotometer. Whole cells were sonicated and mitochondria were solubilized with 0.4% Triton X-100. The reference sample was oxidized with a few grains of ferricyanide and the experimental sample was reduced by the addition of a few crystals of sodium dithionite. The concentration of cytochromes in nanomoles was calculated using the extinction coefficients of Wilson [17]. The low-temperature (77° K) spectrum of purified mitochondria was determined using an Aminco DW–2 uv–visible spectrophotometer fitted with a Dewar flask in which the cuvettes were bathed in liquid nitrogen. The spectrophotometer was operated in the split beam mode using a 1.0 nm slit width. All samples were mixed with glycerol (1:1) prior to freezing. Reduction and oxidation of the sample was achieved as described above. In addition, where indicated, samples were reduced with 10 mM ascorbate and 8 μM tetramethylphenylenediamine.

Electron Microscopy. Cells were prefixed by adding a 6.5% gluteraldehyde fixative to the culture medium in a 1:10 ratio. Cells were then harvested by centrifugation at 750 *g* for less than 1 min at 4°C and fixed in 6.5% gluteraldehyde buffered with Veronal acetate, pH 7.3, at room temperature. Postfixation was in Veronal-acetate-buffered osmium tetroxide (Palade's fluid). Cells were then washed and stained with 1% uranyl acetate and dehydrated by gradual transition through alcohols of increasing concentration. The cells were infiltrated and embedded in Maraglas. Thin sections were examined with a Zeiss EM 9S–2 or a JEOL 100B electron microscope.

Materials. Ethidium bromide (3,8-diamino-5-ethyl-6-phenyl-phenanthridinium bromide) and streptomycin sulfate, both grade B, were purchased from Calbiochem; Triton X–100 was obtained from Rohm and Haas. Dichlorophenolindophenol, grade 1, phenazine methosulfate, bovine serum albumin (fraction five), cytochrome *c* (horse heart type III or VI), were obtained from Sigma; proteose peptone and yeast extract were obtained from Difco and sucrose (density gradient grade) from Schwarz-Mann. Oligomycin was obtained from ICN Pharmaceuticals Inc. and dissolved in 95% ethanol prior to use. Diphenylamine was obtained from Pfaltz and Bauer Inc.

Results

Effect of Ethidium Bromide on Mitochondrial Function

Previously, we reported [11] that after addition of ethidium bromide to cultures of slime mold amoebae, growth continued for 1 or 2 generations, at which time cell division ceased. The amount of protein in these culures, however, did continue to increase such that the protein per cell in the cultures treated with ethidium bromide for 5 days was double that of control cells (Table I). Previously [12] we had reported that the amount of mitochondrial protein per mg cell protein had increased over 50% after treatment with ethidium bromide. Consequently, the amount of mitochondrial protein per cell is nearly three times greater after 5 days in ethidium bromide. The data of Table I also confirm published reports [18, 19] that ethidium bromide blocks the replication of mitochondrial DNA in the slime mold. The amount of DNA present in mitochondria essentially free of nuclear or lysosomal contamination from ethidium-bromide-treated cultures was decreased 50% as compared to mitochondria from control cells. This result indicates that ethidium bromide blocks DNA replication almost immediately and that the remaining mitochondrial DNA is stable over the time studied. As a further result of treatment with ethidium bromide, the cyanide-sensitive respiration of

TABLE I. Effects of Ethidium Bromide on Slime Molds[a]

		Control	Ethidium bromide	Change (%)
Total Protein	mg/10^8 cells	6.4	13.4	+109
Mito. Protein	mg/10^8 cells	1.41	4.09	+190
DNA	μg/mg mito. protein	6.36	3.38	−49
Succinate dehydrogenase	nmoles/min/mg cell protein	34.3	61.2	+78
Succinate dehydrogenase	nmoles/min/mg mito. protein	192	168	Insig
Succinate dehydrogenase	nmoles/min/μg mito. DNA	30.2	49.7	+65
Whole cell respiration	natoms O/min/mg	34.1	12.9	
$+CN^-$	natoms O/min/mg	8.5	7.7	Insig
Cyanide-sensitive	natoms O/min/mg	25.6	5.2	−75

[a] Cells were grown in the presence of 10 μg/ml of ethidium bromide for 5 days. Mitochondria were prepared, DNA content determined, and enzymes assayed as described under Materials and Methods. Each value is the average of three separate determinations.

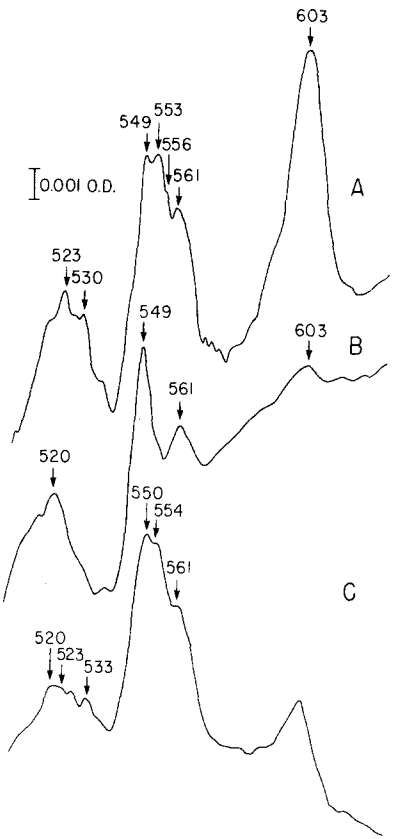


Figure 1. Low-temperature (77°K) reduced–oxidized difference spectra of purified mitochondria from, trace A; control cells; trace B: cells treated with ethidium bromide for five days; trace C: cells that had been washed free of ethidium bromide and allowed to recover for 3 days. Samples were reduced with dithionite and oxidized with ferricyanide. Protein concentration was 2 mg/ml.

whole cells was decreased by 75% (Table I). This result indicates that the previously reported decreases in respiratory chain activity of purified mitochondria have a profound effect on whole cell physiology and may explain the lack of growth in ethidium bromide. The resulting lowered level of energy production may be sufficient to maintain cell viability and some protein synthesis, but insufficient for the cells to undergo cell division.

The observed decreases in respiratory chain activity after ethidium bromide treatment are reflected in concomitant losses of mitochondrial cytochromes. Previously, we reported that the total amounts of

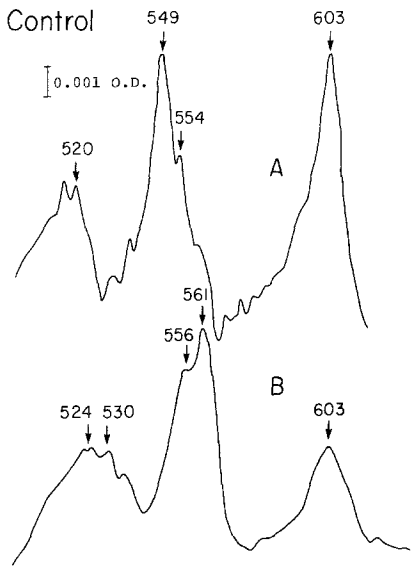


Figure 2. Low-temperature (77°K) reduced–oxidized difference spectra of purified mitochondria from control cells. Trace A: Sample was reduced with ascorbate-tetramethylphenylenediamine and oxidized with ferricyanide. Trace B: Dithionite reduced with the reference cuvette containing mitochondria reduced with ascorbate-tetramethylphenylenediamine. Protein concentration was 2 mg/ml.

cytochromes a–a_3 and b were decreased 73% and 38%, respectively, while that of cytochromes c–c_1 was increased 69% above control levels after treatment with ethidium bromide. These changes in cytochrome content have now been further characterized by low-temperature difference spectroscopy, which has permitted the resolution of cytochromes c and c_1 and revealed the presence of two b-type cytochromes in slime mold mitochondria (Fig. 1). In the trace of control mitochondria reduced with dithionite (Fig. 1A), the absorption maxima at 549 and 554 nm are presumably due to cytochromes c and c_1 while those at 556 and 561 nm represent b-type cytochromes. Further evidence for these assignments is apparent in Fig. 2, in which control mitochondria were first reduced with ascorbate-tetramethylphenylenediamine (trace A) and then reduced with dithionite using the ascorbate reduced mitochondria in the reference cuvette (trace B). In trace A, the absorption band at 603 nm due to cytochromes a–a_3 is apparent as well as the two bands at 549 and 554 nm due to cytochromes c and c_1. The presence of two bands at 520 and 525 nm is also apparent in the beta region of the spectrum. Since ascorbate tetramethylphenylenediamine acts to reduce the respiratory chain at the

level of cytochromes *c* and c_1, further addition of dithionite using the ascorbate-reduced mitochondria as the reference will show the reduction of only *b* cytochromes. Figure 2, trace B reveals two distinct peaks at 556 and 561 nm, which most likely represent two different forms of cytochrome *b*.

Mitochondria purified from slime mold cultures treated with ethidium bromide for 5 days were also examined by low-temperature difference spectroscopy. As seen in Fig. 1, trace B, the bands at 603 and 561 nm due to cytochromes a–a_3 and presumably one of the *b*-type cytochromes were greatly reduced as compared to the control mitochondria. The cytochrome *c* region of the spectrum was also altered significantly in the mitochondria after ethidium bromide treatment, revealing an almost symmetrical peak at 549 nm due to cytochrome *c* with only a slight shoulder at 554 nm due to cytochrome c_1. Reduction of these mitochondria with ascorbate further emphasized the prominence of the band due to cytochrome *c* and the pronounced loss of cytochrome c_1 (Fig. 3, trace A). The beta region of the

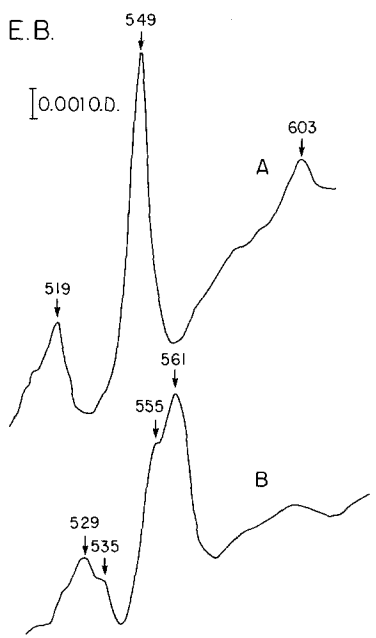


Figure 3. Low-temperature (77°K) reduced–oxidized difference spectra of purified mitochondria from cells treated with ethidium bromide for 5 days. Trace A: Sample was reduced with ascorbate-tetramethylphenylenediamine and oxidized with ferricyanide. Trace B: Dithionite reduced with the reference cuvette containing mitochondria reduced with ascorbate-tetramethylphenylenediamine. Protein concentration was 2 mg/ml.

spectrum also reveals a single symmetrical band at 519 nm due to cytochrome *c*. The presence of two *b*-type cytochromes in the mitochondria from ethidium-bromide-treated cultures was apparent when the dithionite reduced mitochondria were analyzed using ascorbate-reduced mitochondria as the reference. These spectra indicate that the cytochrome *b* absorbing at 561 nm is lowered approximately 30% while that absorbing at 555–556 nm is reduced by only 10%. The trace B spectrum obtained after dithionite reduction (Fig. 1, trace B) also indicates a pronounced decrease in absorbance at 561 nm.

Recovery of Slime Molds from Ethidium Bromide Treatment

The effects of ethidium bromide on slime mold amoebae appear to be completely reversible. When cells were removed from the ethidium bromide medium by low-speed centrifugation, thoroughly washed to remove the drug, and resuspended in fresh nutrient medium, growth and cell division began immediately without a detectable lag. Examination of the growing cells by difference spectroscopy at room temperature (Fig. 4) indicated that the cytochrome content did not return to the control levels

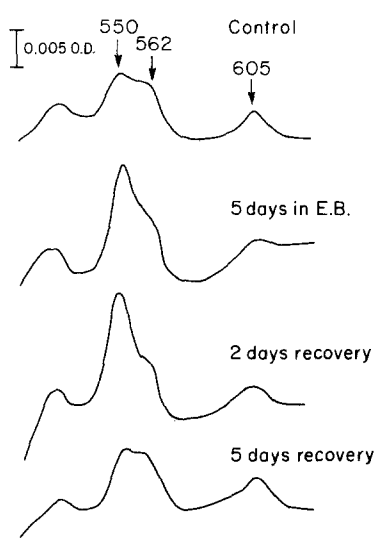


Figure 4. The dithionite-reduced, ferricyanide-oxidized absorption spectra at room temperature of whole cells solubilized in 0.4% Triton X–100 obtained from, upper trace: control cells; second trace: cells that had been treated with ethidium bromide for 5 days; third trace: cells that had been allowed to recover for 5 days. Cuvettes contained between 4 and 6 mg protein.

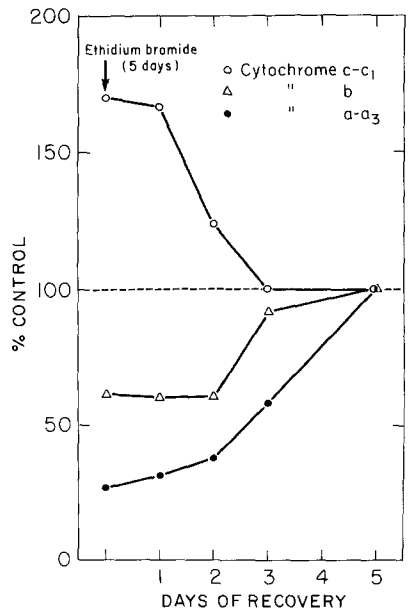


Figure 5. Time course of the recovery of cytochromes based on room-temperature difference spectroscopy of purified mitochondria. Control values for cytochromes c–c_1, cytochrome b and cytochromes a–a_3 were 0.307, 0.578, and 0.242 nmoles/mg protein, respectively, calculated as described in Materials and Methods.

until 5 days after removal of the ethidium bromide despite the immediate return to the normal growth rate.

Spectral analysis of purified mitochondria from cells after removal of ethidium bromide confirmed the lag in recovery of the mitochondrial cytochromes (Fig. 5). The amount of both cytochromes a–a_3 and the b-type cytochromes remained nearly constant for the first two days after removal of ethidium bromide and then rapidly increased reaching control levels at different times. Cytochromes a–a_3, the most severely reduced by ethidium bromide treatment, did not recover completely until 5 days after removal, while cytochrome b reached the control level after 3 days. Likewise, the amount of cytochrome c rapidly decreased once the ethidium bromide was removed, reaching control levels within 3 days. Low-temperature spectroscopy of the purified mitochondria 3 days after ethidium bromide removal confirmed the 50% recovery of the band at 603 nm due to cytochromes a–a_3, and the complete recoveries of the bands at 561 and 549 nm of cytochromes b and c, respectively (Fig. 1, trace C). The prominent peak at 554 nm due to cytochrome c_1 has also reappeared and returned to the control level.

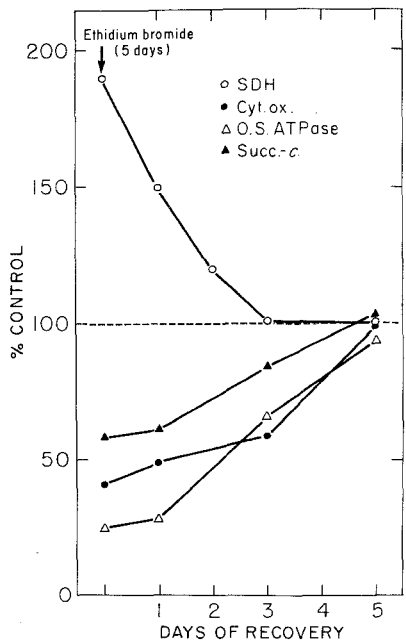
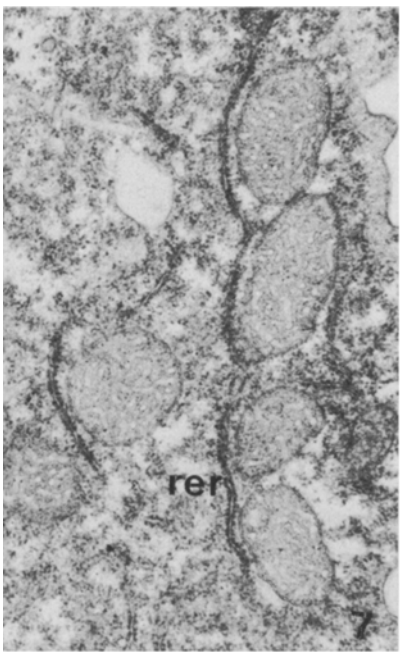


Figure 6. Time course of the recovery of mitochondrial enzymes expressed in terms of percent of control. Succinate dehydrogenase activity was assayed in whole cells solubilized in 0.4% Triton X–100. All other enzyme activities were monitored in purified mitochondria. Control values were for succinate dehydrogenase (34.3 nmoles of substrate oxidized/min/mg whole cell protein), cytochrome oxidase (1.36 k/mg mitochondrial protein), oligomycin-sensitive ATPase (0.495 μM Pi/min/mg mito. protein), and succinate–cytochrome-*c* reductase (186 nmoles/min/mg mito. protein).

In agreement with the spectral data, the enzymatic activities of cytochrome oxidase and succinate–cytochrome-*c* reductase returned to the control levels with similar kinetics as the cytochromes (Fig. 6). The activity of both enzymes increased very little during the first day and then increased rapidly reaching the control level after 5 days. The activity of oligomycin-sensitive ATPase also remained unchanged the first day after removal of ethidium bromide, then gradually increased reaching control levels within 5 days. Figure 6 also shows the specific activity of succinate dehydrogenase assayed in whole cells. Previously, we reported that the activity of succinate and NADH dehydrogenases calculated per mg of total cell protein had nearly doubled in whole cells maintained in ethidium bromide for 5 days, despite the identical activity of these enzymes in purified mitochondria from control and ethidium-bromide-treated cells. The specific activity of succinate dehydrogenase in whole cells decreased rapidly, reaching control levels within 3 days after removal of the drug.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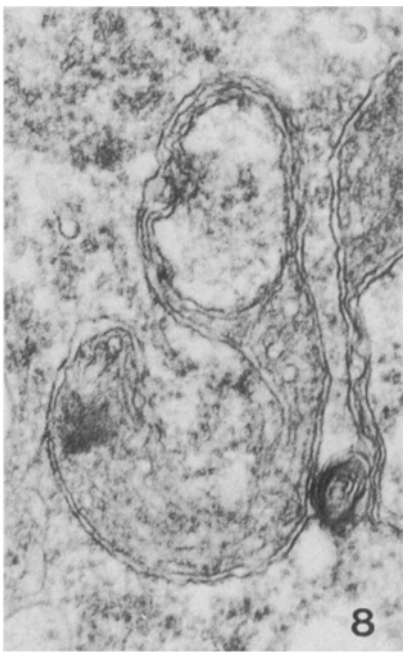
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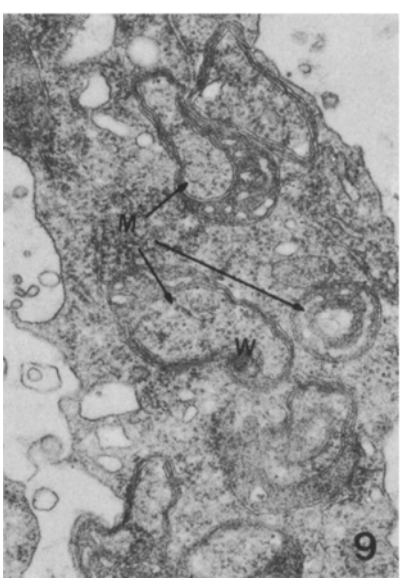
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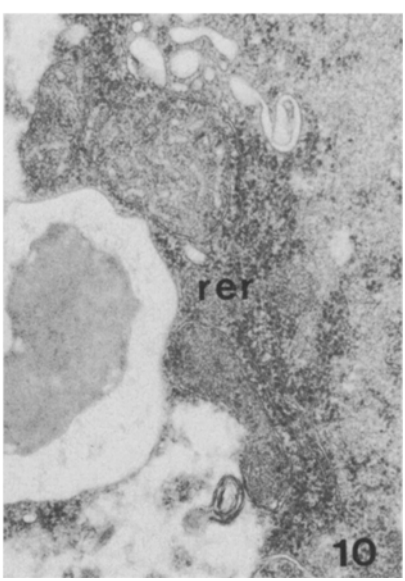
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Electron Microscopy

Ultrastructural studies of control and ethidium-bromide-treated cells were performed to investigate the effects of the drug on mitochondrial morphology. Control cells in the vegetative stage of growth contain many spherical to ovoid-shaped mitochondria with numerous cristae (Fig. 7). In addition, rough endoplasmic reticulum is abundant and often seen closely apposing each mitochondrion. After 2 days in ethidium bromide many mitochondria have become elongated and twisted, while the number of cristae appear reduced (Fig. 8). Furthermore, it appears that mitochondria have become twisted upon themselves in the process of forming whorled membranous profiles. After 5 days of exposure to ethidium bromide, even more striking morphological effects are observed. In general, cells appear to lack the organized appearance of control cells and many whorled membrane figures are observed in all cells (Fig. 9). Rough endoplasmic reticulum is no longer prominent, while structures still identifiable as mitochondria appear twisted and without extensive cristae (Fig. 9).

Biochemical evidence described above has demonstrated that when the cells are washed free of ethidium bromide and allowed to grow in nutrient media for 3 days, the levels of mitochondrial enzymes have almost completely recovered from the effects of the drug. Electron micrographs of such cells show that most mitochondria are ultrastructurally indistinguishable from those of control cells (Fig. 10); however, the general appearance of the cells is still morphologically abnormal (Fig. 11). All cells at this stage of recovery have a large proportion, approximately 30–75% of their volume, compartmentalized in a single large vacuole, within which is an amorphous material resembling that found in lysosomes (Fig. 11). A single membrane within the vacuole encloses many abnormal membrane structures. Whorled membrane figures are very obvious at this time and are almost always found within the large inner compartment (Fig. 11).

Figure 7. Thin section through a vegetative amoeba reveals the presence of rough endoplasmic reticulum (rer) adjacent to each mitochondrion. Mitochondria contain numerous cristae. ×25,000.

Figure 8. After 2 days in ethidium bromide, mitochondria appear greatly twisted. ×70,000.

Figure 9. Altered mitochondria (M) lacking inner membrane infoldings after ethidium bromide treatment for 5 days. A whorl (w) appears to be forming within a mitochondrion. ×28,000.

Figure 10. Three days after removal of ethidium bromide, rough endoplasmic reticulum (rer) is again in close juxtaposition to mitochondria containing numerous cristae. ×40,000.

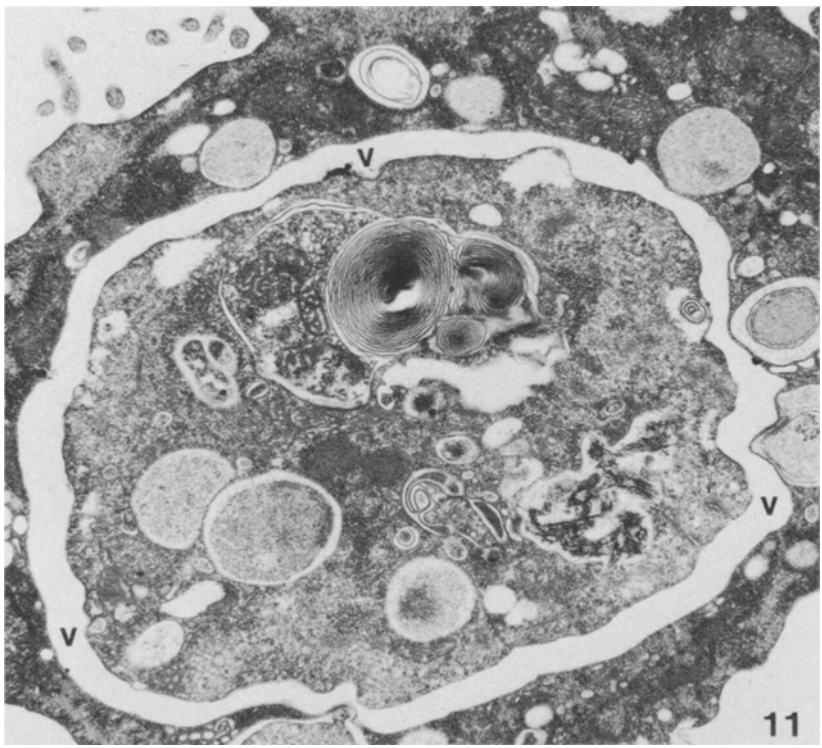

Figure 11. Intact cell 3 days after removal of ethidium bromide. Large vacuole (V) observed in all cells. Membranous whorls present. x 20,000.

Rough endoplasmic reticulum is again present in abundance in close juxtaposition to mitochondria (Fig. 10).

Discussion

The present report is a continuation of our previous studies concerning the effects of ethidium bromide on mitochondria of the cellular slime mold, *Dictyostelium discoideum*. After addition of ethidium bromide to cultures of slime mold amoebae, continued synthesis of cytochrome oxidase and oligomycin-sensitive ATPase ceased, while that of the cytochrome b–c_1 complex of the respiratory chain was partially inhibited. As a consequence of the losses in respiratory activity, cell division continued for only one or two generations; however, the amount of protein per cell continued to increase, doubling after 5 days in ethidium bromide, a time equivalent to eight generations. Significantly, during this time in ethidium bromide, the

amount of mitochondrial protein per mg of cell protein had also increased over 50%. This conclusion is based on the observation that the activities of succinate and NADH dehydrogenases were identical in purified mitochondria from control and ethidium-bromide-treated cells despite the twofold increase in the activity of these enzymes in whole cells after treatment with the drug. Hence, new mitochondrial membranes with identical amounts of the flavoproteins must continue to be formed after cell division had ceased. Furthermore, our previous studies [12] had indicated that after isopyknic centrifugation in a sucrose density gradient, the most highly purified mitochondrial fractions obtained from either control cultures or those treated with ethidium bromide for 5 days had the same buoyant density. This observation also suggested that despite the approximately 80% decrease of cytochromes a–a_3 and oligomycin-sensitive ATPase activity as well as the partial loss of cytochrome b in mitochondria obtained from slime mold cells grown in the presence of ethidium bromide, no corresponding change in the mitochondrial membrane occurred to affect the density of the over-all structure.

Despite the continued synthesis of mitochondrial membranes in the presence of ethidium bromide, the DNA content per mg of purified mitochondria has decreased 50%. This result suggests that ethidium bromide may immediately block the replication of mitochondrial DNA assuming that the amount of mitochondrial protein doubles during the one cell division that occurs after the addition of the drug. Interestingly, after treatment with ethidium bromide the amount of mitochondrial DNA per cell can be calculated to increase slightly in parallel with the threefold increase in mitochondrial protein per cell (Table I). These data suggest that as a consequence of ethidium bromide treatment, the regulation of both cell and organelle growth is disrupted. Both the total amount of mitochondrial protein and DNA increase in each cell to a greater extent than the total protein per cell increases. These changes may be an attempt by the cell to compensate for the loss of ATP production when synthesis of the respiratory chain has been blocked by ethidium bromide.

In this report, we have also analyzed more closely the changes in cytochrome content that result from the addition of ethidium bromide. Previously, it was shown that mitochondria purified from cells treated with ethidium bromide for 5 days have reduced levels of cytochromes a–a_3 and b and increased levels of cytochromes c–c_1 as determined by difference spectroscopy at room temperature [12]. In the present study, cytochromes c and c_1 as well as two b-type cytochromes have been resolved by low-temperature difference spectroscopy (Figs 1–3). Significantly, treatment with ethidium bromide results in an 80% decrease in the amount of cytochrome c_1 with a concomitant twofold increase in the amount of

cytochrome *c*. Recently, Ross and Schatz [20] have reported that the apoprotein of cytochrome c_1 is synthesized on extramitochondrial ribosomes; however, formation of the holo-cytochrome c_1 in growing yeast cells appears to require mitochondrial protein synthesis. The present results further suggest that the elaboration of complete cytochrome c_1 or its assembly into the membrane may also require mitochondrial transcription.

The presence of two *b*-type cytochromes has now been established in mitochondria of the slime mold. Surprisingly, both cytochromes b_{556} and b_{561} are still present after treatment with ethidium bromide, although the total cytochrome *b* content has decreased 30–50%. Examination of the spectra suggests that cytochrome b_{561} is apparently reduced more by treatment with ethidium bromide than is cytochrome b_{556}; however, it is difficult to quantitate the absolute decrease. Recently the intramitochondrial site of cytochrome *b* synthesis has been demonstrated with purified preparations of cytochrome *b* with an absorption maximum at 562 nm, from both Neurospora [21] and yeast [22] while genetic evidence suggests that yeast mitochondrial DNA also codes for cytochrome *b* [23]. Hence, it is difficult, in our present state of knowledge, to explain the observation that the content of cytochrome *b* and succinate–cytochrome-*c* reductase activity are not as severely reduced in slime mold mitochondria by ethidium bromide treatment as are cytochrome oxidase and oligomycin-sensitive ATPase, two other enzyme complexes also requiring both mitochondrial translation and transcription (Figs 5 and 6).

All of the effects of ethidium bromide described above are completely reversible. after removal of the drug, cell division began immediately; however, a pronounced lag was observed before the content of mitochondrial cytochromes or enzymatic activities began to increase. By contrast, the elevated levels of cytochrome *c* and succinate dehydrogenase decreased rapidly without a lag. The different time course by which the various components returned to the control level confirms previous suggestions that the mitochondrial inner membrane is assembled in an asynchronous manner [24].

In the present study, the ethidium-bromide-induced alterations in cellular morphology were investigated by electron microscopy. After addition of ethidium bromide, mitochondrial profiles became progressively more elongated and twisted while the cristae were almost completely absent after 5 days. These changes in mitochondrial morphology correlate well with the large decrease in the activity of inner membrane components of the respiratory chain. Similar effects of ethidium bromide on mitochondrial morphology have been described in HeLa cells [6, 25, 26], chinese hamster fibroblasts [27], and mouse L cells

[6, 7]. Likewise, treatment with chloramphenicol, a specific inhibitor of mitochondrial protein synthesis [28], also results in a damaged appearance of mitochondria [6, 29, 30], suggesting that the presence of proteins synthesized on mitochondrial ribosomes is required for the maintenance of mitochondrial structure.

Ethidium bromide treatment also appears to affect other cellular structures as well as the mitochondria. Control cells contain well-developed rough endoplasmic reticulum, some of which can be observed in close apposition to the mitochondria (Fig. 7). After exposure to the drug for 5 days, the amount of rough endoplasmic reticulum is reduced to insignificant levels and none is seen adjacent to any of the mitochondrial profiles.

Three days after removal of ethidium bromide, the cells appear to have recovered morphologically. Most mitochondria once again appear spheroid with well-organized cristal membranes (Fig. 10) and are adjacent to rough endoplasmic reticulum. At this stage of recovery all cells appear to have compartmentalized their damaged membranes and organelles within a single large vacuole which sometimes occupies 75% of the cell volume (Fig. 11). This structure appears to consist of a vacuole enclosed by a single membrane and surrounded by an amorphous material which in turn is enclosed in a unit membrane. The material between the two unit membranes appears similar to that found in lysosomes. Preliminary observations with the Gomori stain procedure indicate the presence of acid phosphatase activity within the vacuole. These recovery vacuoles vary in size but are quite distinct from structures found in control cells, and they should not be confused with the osmoregulating vacuole present in vegetative amoebae. The compartmentalization of damaged membranes in these vacuoles suggests that this may be a more efficient way of removing or degrading them, and we have not ruled out the possibility that this vacuole fuses with the cell plasma membrane "secreting" damaged cell material. Alternatively, this vacuole may simply represent a phagocytized cell, as these amoebae are known to cannibalize other cells when starved. Further studies of *Dictyostelium discoideum* undergoing recovery from the effects of ethidium bromide are in progress to clarify further these observations.

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