Effects of Ethidium Bromide on the Respiratory Chain and Oligomycin-sensitive Adenosine Triphosphatase in Purified Mitochondria from the Cellular Slime Mold Dictyostelium discoideum*

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SUMMARY

Mitochondria were isolated from the cellular slime mold, Dictyostelium discoideum, and partially purified by sucrose density gradient fractionation. The most purified mitochondrial fraction from the gradient contained essentially no contaminating lysosomes and minimal amounts of contaminating peroxisomes as determined by the marker enzymes N-acetylglucosaminidase and catalase. A mitochondrial fraction with the same amount of lysosomal and peroxisomal contamination was also isolated from cells which had been treated with ethidium bromide for 5 days. The most purified mitochondrial fraction from control and ethidium bromide-treated cells had an identical buoyant density of 1.181 to 1.182 g per ml, suggesting that treatment with the drug does not result in any drastic structural changes in the mitochondrial membrane which would affect its density. In the purified mitochondria from ethidium bromide-treated cells, the content of cytochromes a-a₃ was decreased over 80% and that of cytochrome b by 35%. The activities of cytochrome oxidase and oligomycin sensitive ATPase were reduced approximately 80%, whereas the activity of succinate-cytochrome c reductase was decreased 50%. By contrast, the specific activities of NADH and succinate dehydrogenases were identical in the purified mitochondria from control and ethidium bromide-treated cells. Previously, we had reported that the specific activities of these two enzymes had nearly doubled in whole cells maintained in ethidium bromide for a time equivalent to six or seven generations after growth had stopped (STUCHELL, R. N., WEINSTEIN, B. I., AND BEATTIE, D. S. (1973) Fed. Eur. Biochem. Soc. Lett. 37, 23-26). These results suggest that continued formation of new mitochondrial membranes, with an identical complement of succinate and NADH dehydrogenases, must occur despite the cessation of cell growth which occurs as a result of the ethidium bromide induced loss of mitochondrial enzymes. Consequently, the amount of mitochondria, or mitochondrial protein per cell, calculated from the activity of NADH and succinate dehydrogenases has increased nearly 50%. Possible models to explain the control of mitochondrial biogenesis are discussed to explain these results.

Ethidium bromide and the acridine dyes which intercalate between the bases of DNA (1) have been shown to inhibit selectively the replication and transcription of mitochondrial DNA (2, 3). In an early study, Slonimski (4) demonstrated that the addition of ethidium bromide to growing cultures of yeast caused the irreversible conversion of wild type cells to the cytoplasmic "petite" mutation which is characterized by the complete loss of mitochondrial cytochromes. By contrast, addition of ethidium bromide to cultures of mammalian cells produces changes which are completely reversible. Treatment of human fibroblast cultures (5), L cells (6, 7) and SV40-transformed cells (8) with ethidium bromide for several days caused a drastic reduction in the cytochrome a-a₃ content and a variable decrease in the level of cytochrome b. Morphologically, changes in mitochondrial structure including a reduction in cristae have been observed in cultures of both L cells (6) and HeLa cells (9) treated with ethidium bromide for several days.

The cellular slime mold Dictyostelium discoideum is an excellent organism for studies of both mitochondrial biogenesis and the role of mitochondria during cellular differentiation. The slime mold grows as a single cell amoeba when supplied with appropriate nutrients. Under controlled conditions, the amoeba aggregate into a multicellular mass and synchronously differentiate into more than one distinct cell type. Previously we reported (10) that after addition of ethidium bromide to cultures of slime mold amoebae, growth continued for only one or two more generations. The specific activity of cytochrome oxidase decreased immediately after addition of the drug, while the specific activity of succinate-cytochrome c reductase decreased more slowly over a period of time equivalent to six or seven generations. These results had been anticipated, since earlier
studies with yeast (11) and mammalian cells in tissue culture (6-8) had suggested that ethidium bromide causes a primary effect on the formation of cytochromes a-d, and a more gradual, perhaps secondary effect on the formation of cytochrome b. Presumably, the ability of the cells to grow and divide was lost when energy production by the respiratory chain became limiting due to the loss of these essential cytochromes. By contrast, the specific activity of the two mitochondrial membrane-bound flavoproteins, succinate and NADH dehydrogenases, gradually began to increase when the cells in ethidium bromide had stopped dividing. The specific activity of both these enzymes had reached a value nearly twice that of the control cells after 5 days.

In order to investigate more fully these changes in the mitochondrial respiratory chain induced by ethidium bromide, especially the increase in the flavoprotein dehydrogenases, it was necessary to develop a method to obtain purified mitochondria from the slime mold. A comparison of the activity of several enzymes in mitochondria obtained from both control and ethidium bromide treated cultures has indicated that treatment with the drug results in an 80% decrease in both cytochrome oxidase and oligomycin-sensitive ATPase, and a 50% decrease in succinate-cytochrome c reductase. No change in the activity of either succinate or NADH dehydrogenases in purified mitochondria was observed; however, the amount of mitochondrial protein per cell increased significantly. These results are discussed in terms of different models for the control of mitochondrial biogenesis.

**METHODS**

**Cell Culture—** Axenic cultures of Dictyostelium discoideum strain A3-3 were grown as described previously (10). Cells that were to be treated with ethidium bromide were grown to a density of 0.5 to 1.0 x 10^6 cells per ml before the addition of the drug (10 pg per ml, final concentration). Usually 3 liters of cells were grown at 22-23°C in a 4-liter Erlenmeyer flask wrapped with aluminum foil on a New Brunswick gyratory shaker at 200 rpm. The cells used as controls were in log phase (2 to 3 x 10^6 cells per ml), since previous studies had indicated that slight variations in mitochondrial enzymatic activities occurred as the cells approached stationary phase (1 to 1.5 x 10^6 cells per ml).

**Preparation of Mitochondria—** Cells were harvested by pouring the growth media into 250-ml centrifuge bottles, accelerating the centrifuge to 4000 x g, and then shutting off the centrifuge. The procedures provided a rapid means of collecting cells from large volumes of growth media. The pelleted cells were washed once in cold distilled water and then in a medium containing 0.25 M mannitol, 0.01 M Tris buffer (pH 7.6), and 0.001 M EDTA (Buffer A). For best results, a thick slurry of cells was pipetted into a 20 ml Thomas-Ten Broeck glass-plastic homogenizer and given 8 to 20 twisting strokes by hand. The homogenate was then diluted with 3 volumes of Buffer A and centrifuged at 3000 X g for less than 1 min to collect unbroken cells. The pellet was rehomogenized and recentrifuged at 3000 X g. The rehomogenization and centrifugation procedure was repeated two more times. All of the supernatants were pooled and centrifuged at 750 X g for 10 min. The pellet was discarded and the supernatant was again centrifuged at 750 X g for 10 min. The resulting supernatant was centrifuged at 7700 X g for 15 min. The pellet was resuspended in Buffer A, gently homogenized, and centrifuged at 7700 X g for 15 min. The pellet was then diluted with Buffer A to give a protein concentration of 10 to 15 mg per ml. Three milliliters of the 7700 X g pellet fraction were carefully layered onto a 35 to 60% (w/v) preformed linear sucrose gradient (0.01 M Tris, pH 7.6, 0.001 M EDTA) which had been prepared the previous evening. The gradients were then centrifuged in a Beckman SW 27 rotor at 37,000 rpm for 105 min at 5°C. The gradients were fractionated by piercing the bottom of the tubes and collecting 20 fractions of approximately 1.6 ml each.

**Density Determinations—** The refractive indices (sodium D line) of the sucrose gradient fractions were determined with a Bauch and Lomb (Abbe-type) refractometer at 25°C. The refractive indices were converted to densities from standard tables.

**Enzyme Assays—** N-Acetylglucosaminidase was assayed according to the procedure of Loomis (12) using fractions which had been lysed with 0.04% Triton X-100.

A 1-ml assay mixture containing 0.01 M sodium acetate, pH 5.0, 0.01 M p-nitrophenyl-N-acetyl-b-D-glucosaminide and a 50% decrease in catalase activity representing peroxisomes banded in the gradient containing succinate dehydrogenase activity. The absorbance at 420 nm was determined with a Gilford spectrophotometer. One unit of activity is defined as the production of 1 nmol of p-nitrophenol per min under the above conditions. An absorbance coefficient of 0.016 for the absorbance at 420 nm was measured to be 15.5 x 10^-5 cm^-1.

Cytochrome oxidase, succinate-cytochrome c reductase, succinate dehydrogenase, and NADH dehydrogenase were assayed at 25°C in a Gilford spectrophotometer as described by Kim and Beattey (13).

Catalase was assayed according to the method of Luck (14). The change in absorbance at 240 nm was determined at 25°C. The absorbance coefficient of 43.5 x 10^-5 cm^-1 was used.

ATPase activity was measured at 25°C in a medium containing 3 mM MgCl2, 10 mM ATP, 50 mM Tris, pH 9.0, and approximately 1 mg of mitochondrial protein in a final volume of 1.0 ml as described by Kim and Beattey (13).

Protein was determined by the method of Lowry et al. (15) using bovine serum albumin as standard.

**Spectral Studies—** The visible spectrum of purified mitochondria was determined by difference spectroscopy in a Cary model 15 spectrophotometer. The fractions were collected with small amounts of deoxycholate and reduced with a few grams of dithionite. The reference sample was oxidized with a few grams of ferricyanide. Alternatively, the reference sample was reduced with 10 mM ascorbate and 8 µM tetramethylphenylenediamine.

The concentration of cytochromes in nanomoles was calculated using the extinction coefficients of Kiese (16).

**Materials—** Ethidium bromide, grade B, was purchased from Calbiochem; Triton X-100, was obtained from Rohm and Haas; dichlorophenolindophenol, grade 1, phenazine methosulfate, bovine serum albumin (Fraction V), cytochrome c (horse heart type III or VI), succinate (sodium salt), ascorbate (sodium salt), glucose, manitol, and p-nitrophenyl-N-acetyl-b-D-glucosaminide grade III, were obtained from Sigma; protease peptone and yeast extract were obtained from Difeo; and sucrose (density gradient grade) from Schwarz-Mann.

**RESULTS**

**Preparation of Purified Mitochondrial Fraction—** In our initial attempts to isolate mitochondria from the slime mold by differential centrifugation, the pellet obtained at any centrifugal force from 6000 to 6000 X g was heavily contaminated with lysosomes as determined by N-acetylglucosaminidase or acid phosphatase activity. When the once washed 7700 X g pellet was further subjected to isopyknic centrifugation in a preformed continuous sucrose gradient, a single band of protein was observed coincident with succinate dehydrogenase activity (Fig. 1). In some experiments, a minor band of protein containing succinate dehydrogenase activity was observed near the top of the gradient, possibly due to fragments of mitochondrial membranes damaged during homogenization of the cells. The buoyant density of the most purified mitochondrial fraction was 1.182 ± 0.003 which is in close agreement to published values for mitochondria from other tissues (17, 18). After sucrose gradient centrifugation, over 70% of the activities of the lysosomal enzymes were observed in the pellet with only minor activities in the region of the gradient containing succinate dehydrogenase activity. The remaining activity was present at the top of the gradient and represents enzymes released from disrupted lysosomes. By contrast, catalase activity representing peroxisomes banded in the gradient very close to the mitochondria (Fig. 1).

A quantitative estimate of the degree of contamination of the
mitochondrial fraction obtained by either differential or gradient centrifugation was calculated using marker enzymes for lysosomes and peroxisomes (Table I). The mitochondria in both the 7700 × g pellet and in the gradient fraction of density 1.182 g per ml were significantly purified as indicated by the 9- and 17-fold increases in specific activity of succinate dehydrogenase compared to the homogenate, which in these experiments, is the 600 × g supernatant. In addition, over 18% of the succinate dehydrogenase activity of the homogenate was recovered in the gradient fraction. Although the specific activity of catalase was also increased 4-fold in this same gradient fraction, less than 5% of the homogenate activity was recovered in the most purified gradient fraction. Lysosomes, as indicated by the N-acetylglucosaminidase activity, were concentrated in the 7700 × g pellet; however, the N-acetylglucosaminidase specific activity was decreased significantly in the gradient fraction and only 1.3% of the total activity of the homogenate was in this fraction.

A mitochondrial fraction was also prepared from slime mold cultures which had been treated with ethidium bromide for 5 days. As seen in Fig. 1, the succinate dehydrogenase activity of the mitochondria from the ethidium bromide-treated cells also banded in the sucrose gradient coincidentally with the major band of protein. The buoyant density of the most purified mitochondrial fraction as indicated by the highest activity of succinate dehydrogenase was 1.181 ± 0.002 g per ml. It should be noted that this value is identical to that obtained for purified mitochondria from control cultures of slime mold amoebae.

The degree of contamination by other organelles of the most purified mitochondrial fraction from ethidium bromide treated cultures is presented in Table I. The recoveries of succinate dehydrogenase, catalase, and N-acetylglucosaminidase in the gradient fraction of 1.181 buoyant density were, respectively, 24, 4.5, and 1% of the total activity of the homogenate. Hence, the mitochondrial fraction obtained from ethidium bromide-treated cells has an identical degree of contamination by lysosomes and peroxisomes as does the mitochondrial fraction obtained from control cultures.

Table I

| Fraction                              | Total protein | Succinate dehydrogenase | Catalase | N-Acetylglucosaminidase |
|---------------------------------------|---------------|-------------------------|----------|------------------------|
|                                       |               | Specific activity        | Total activity | Specific activity | Total activity | Specific activity | Total activity |
|                                       | mg            | mmol/min/mg             | mmol/min   | mmol/min/mg          | mmol/min      | mmol/min/mg      | mmol/min      |
| Control                               |               |                         |           |                       |               |                   |               |
| Whole cells                           | 194.5         | 40.5                    | 2,780      | 129.5                 | 9,300         | 695               |
| 600 × g supernatant                   | 19.0          | 14.3                    | 2,450      | 98.5                  | 19,300        | 201               | 39,100        |
| Mitochondria from gradient fraction   | 2.07          | 245                     | 509        | 413                   | 855           | 254               | 526           |
| Ethidium bromide-treated              |               |                         |           |                       |               |                   |               |
| Whole cells                           | 150.6         | 67.5                    | 103        | 91.6                  | 30.1          | 4,530             |
| 600 × g supernatant                   | 16.9          | 15.8                    | 2,380      | 102                   | 15,400        | 113               | 1,910         |
| Mitochondria from gradient fraction   | 2.16          | 265                     | 572        | 323                   | 700           | 21.7              | 46.9          |
The decreased content of cytochrome creased 21% after treatment with ethidium bromide (Table III). The characteristic bands of cytochromes u-u3, and ethidium bromide-treated cells confirmed the decreases in reductase was decreased nearly 50%.

The total amounts of cytochromes a-a3 observed at 605, 562, and 552 nm, respectively, in the purified obtained from both control and ethidium bromide-treated cells. The amount of mitochondrial protein present in 100 mg of whole cells was calculated by dividing the total activity of either succinate or NADH dehydrogenase in the cells by the specific activity of the enzyme in the most purified gradient fraction. The mitochondrial protein content in control cells was determined to be 21.1 mg/100 mg of cell protein using NADH dehydrogenase. By contrast, the mitochondrial protein content of the ethidium bromide-treated cells was 31.5 and 30.5 mg/100 mg of cell protein calculated using these enzymes.

Previously (10) we reported that the addition of ethidium bromide to slime mold cultures immediately blocked the formation of cytochromes a-a3, determined either spectrophotometrically or enzymatically. The synthesis of cytochrome b, determined either spectrophotometrically or enzymatically, using succinate-cytochrome c reductase, was also inhibited by ethidium bromide treatment but at a lower rate. The activity of these enzyme complexes was compared in purified mitochondria obtained from both control and ethidium bromide-treated cells (Table II). The specific activity of cytochrome oxidase was decreased 82%, while the specific activity of succinate-cytochrome c reductase was decreased nearly 50%.

Spectral analyses of the purified mitochondria from control and ethidium bromide-treated cells confirmed the decreases in enzymatic activity of these two segments of the respiratory chain. The characteristic bands of cytochromes a-a3, b, and c-c1 were observed at 906, 562, and 552 nm, respectively, in the purified mitochondria (Fig. 3). The total amounts of cytochromes a-a3 and b were decreased 82% and 35%, while that of c-c1 was increased 21% after treatment with ethidium bromide (Table III).

The decreased content of cytochrome b measured at 562 nm was more apparent when the dithionite-reduced mitochondria were analyzed using ascorbate-tetramethylphenylenediamine reduced mitochondria in the reference cuvette (Fig. 4, bottom panel).

TABLE II

| Specific activity                     | Control                  | Ethidium bromide | P      |
|--------------------------------------|--------------------------|------------------|--------|
| Succinate dehydrogenase (nmol/min/mg)|                          |                  |        |
| Whole cells                          | 42.0 ± 1.3               | 71.6 ± 6.6       | <0.02  |
| Mitochondria                         | 203 ± 16.7               | 235 ± 31.6       | Insufficient |
| NADH dehydrogenase (nmol/min/mg)     |                          |                  |        |
| Whole cells                          | 0.613 ± 0.014            | 0.884 ± 0.028    | <0.01  |
| Mitochondria                         | 2.71 ± 0.18              | 2.90 ± 0.17      | Insufficient |
| Succinate-cytochrome c reductase (nmol/min/mg) | |                  |        |
| Whole cells                          | 86.9                     | 43.5             | <0.02  |
| Mitochondria                         | 190 ± 19.2               | 194.5 ± 12.8     | <0.01  |
| Cytochrome oxidase (k/mg)            |                          |                  |        |
| Mitochondria                         | 3.33 ± 0.47              | 0.649 ± 0.138    | <0.01  |

FIG. 2. The dithionite-reduced ferricyanide-oxidized absorption spectra of purified mitochondria obtained from control and ethidium bromide-treated slime molds. Top trace, mitochondria (3.4 mg per ml) from control cells; bottom trace, mitochondria (3.05 mg per ml) from cells treated with ethidium bromide for 5 days.

Once the purity of the mitochondrial fractions obtained from both control and ethidium bromide-treated cells had been established and shown to be comparable, the activities of several enzymes and enzyme complexes of the mitochondrial respiratory chain were determined. As seen in Table II, the specific activities of succinate and NADH dehydrogenases, were significantly higher (144 to 166%) in whole cells of ethidium bromide-treated as compared to control cultures. No significant differences, however, were observed in the specific activity of either enzyme in the purified mitochondrial fraction from control and ethidium bromide-treated cells. The enzymes were assayed as described under “Methods” in whole cells lysed with Triton X-100 or in mitochondria obtained from the most purified fraction of the sucrose gradient. Each value is the mean ± the standard error of the mean of six different experiments. Treatment with ethidium bromide was for 5 days. Probability values were calculated by Student’s t test.
Inhibition by oligomycin (%)

**Table IV**

**Effect of ethidium bromide on the MgK+-stimulated ATPase of purified slime mold mitochondria**

| ATPase    | Control             | Ethidium bromide                  | p  |
|-----------|---------------------|-----------------------------------|----|
| ATPase    | 1.06 ± 0.15         | 0.357 ± 0.04                      | <0.02 |
| + Oligomycin | 0.206 ± 0.02       | 0.117 ± 0.06                      | N.S.  |

Inhibition by oligomycin (%)

| ATPase | 80.6 | 50.5 |

ATPase was assayed as described under "Methods" using the most purified mitochondrial fraction from the gradient. Where indicated 10 μg of oligomycin were added. Treatment with ethidium bromide was for 5 days.

Slime mold mitochondria was observed at pH 9.0. Oligomycin, added at a final concentration of 10 μg per ml also had maximum inhibitory effects when the enzyme was assayed at pH 9.0. In separate experiments (data not shown), it was determined that maximum inhibition was obtained at oligomycin concentrations of 5 μg per ml when 0.5 to 1 mg of protein was used in the assay. The addition of the uncoupler, dinitrophenol, did not cause any stimulation of ATPase activity in purified slime mold mitochondria.

The total ATPase activity of purified mitochondria treated with ethidium bromide was decreased 66% compared to mitochondria obtained from control cells (Table IV); however, the oligomycin-resistant ATPase activity is identical in the two types of mitochondria. This result indicates that the activity of the oligomycin-sensitive ATPase representing the terminal steps of oxidative phosphorylation is actually reduced almost 80% in the purified mitochondria from ethidium bromide-treated cells.

**Discussion**

In our initial studies of mitochondrial biogenesis in the cellular slime mold Dictyostelium discoideum (10), we made the unexpected observation that the specific activity of the two mitochondrial membrane-bound flavoproteins, succinate and NADH dehydrogenases, in whole cells increased nearly 2-fold in cultures treated with ethidium bromide for 5 days. Concomitantly, a
loss of cytochrome oxidase and succinate-cytochrome c reductase activities was observed. It should be noted that the formation of NADH and succinate dehydrogenases has been shown to require proteins synthesized only on cytoplasmic sensitive cytoplasmic ribosomes (13, 20). The increased specific activity of these two enzymes in whole cells treated with sufficient ethidium bromide to block further mitochondrial DNA synthesis as well as mitochondrial transcription can be explained by two hypotheses. First, it can be assumed that the formation of new mitochondrial membranes continues despite the cessation of cell growth such that each cell now contains more mitochondria or mitochondrial protein. An increased number of mitochondria per cell (or amount of mitochondrial membrane protein per cell) containing a normal complement of membrane-bound enzymes, which do not require either mitochondrial transcription or translation for their synthesis, would be manifest as a greater specific activity of these mitochondrial membrane-bound enzymes per cell. Alternatively, it can be assumed that "abnormal" mitochondrial membranes containing greater amounts of these membrane-bound enzymes are formed when mitochondrial transcription is blocked by ethidium bromide.

To distinguish between these two possibilities, it was necessary to obtain highly purified mitochondria from both control and ethidium bromide-treated cells and to compare the activities of these various enzymes. A mitochondrial fraction containing essentially no contaminating lysosomes and minimal amounts of contaminating peroxisomes was obtained by sucrose density gradient fractionation. Furthermore, the mitochondrial fraction obtained from ethidium bromide-treated cells using the same methods contained exactly the same amount of contamination by lysosomes and peroxisomes. Hence, we were able to compare with confidence the activities of several enzymes and enzyme complexes of the mitochondrial inner membrane in the purified mitochondrial fraction obtained from both types of cells.

After treatment with ethidium bromide, two enzyme complexes of the respiratory chain, cytochrome oxidase and succinate-cytochrome c reductase, were reduced by the same amount in the purified mitochondria as in the whole cells. Spectral analysis confirmed that mitochondria obtained from slime mold cultures treated with ethidium bromide for 5 days contained 82% less cytochrome a-a3 and 33% less cytochrome b than mitochondria from control cultures. Similar changes in cytochrome content induced by ethidium bromide have been reported for human fibroblast cultures (5), L cells (6, 7) HeLa cells (9), and SV40-transformed cells (8).

Mitochondria obtained from slime mold cells treated with ethidium bromide also contained significantly reduced levels of oligomycin-sensitive ATPase activity. Tzagoloff et al. (19) have established that certain hydrophobic membrane proteins of the ATPase complex of yeast are synthesized within the mitochondria; however, it has not been established whether the mRNA for these proteins is transcribed from mitochondrial DNA. The observation that the oligomycin-sensitive ATPase activity is decreased 80% in mitochondria obtained from ethidium bromide-treated slime mold cells plus the earlier reports that total ATPase activity is reduced in yeast mitochondria with a partial or total loss of mitochondrial DNA (21) suggests that the genetic information for these proteins may be in the mitochondria DNA.

A surprising finding in this study was the observation that no change in the buoyant density of the mitochondria occurred after treatment of slime mold cultures with ethidium bromide for 5 days despite the extensive loss of many enzymatic activities. After isopyknic centrifugation in a sucrose density gradient, the most highly purified mitochondrial fraction i.e. that with the greatest activity of succinate dehydrogenase and a minimum amount of catalase, banded at a buoyant density of 1.181 to 1.182 g per ml. A comparison of at least four different preparations from both control and 5-day ethidium bromide-treated cells indicated that there was no significant difference in the buoyant densities of the purified mitochondria. This observation suggests that despite the approximately 80% decrease of cytochromes a-a3 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 changes in the mitochondrial membrane occurred to affect the density of the over-all structure. A similar conclusion was reported by Packer et al. (21) in a study of mitochondrial membranes obtained from yeast made respiratory deficient with ethidium bromide. The loss of cytochromes and decrease in oligomycin-sensitive ATPase activity was not accompanied by any changes in the membrane structure as observed in freeze-fracture electron micrographs. The size and distribution of membrane particles was identical indicating that the mitochondrial membranes obtained from wild type cells and the mutant without mitochondrial DNA were not significantly different. These results, plus our observation that treatment with ethidium bromide caused no change in the buoyant density of slime mold mitochondria, suggest that the ethidium bromide-induced loss of respiratory chain components plus oligomycin-sensitive ATPase does not result from any drastic structural changes in the mitochondrial membrane. Perhaps ethidium bromide inhibits the synthesis of only selected subunits of these enzyme complexes which are essential for catalytic function of cytochrome oxidase (19, 22), oligomycin-sensitive ATPase (19), and Complex III of the respiratory chain which contains cytochrome b (23, 24).

Further evidence which indicates that the mitochondrial membranes from both control and ethidium bromide-treated cells are identical was obtained when the activities of succinate and NADH dehydrogenases were compared in the purified mitochondria. No significant difference in the activity of either of these enzymes was observed in the purified mitochondrial fraction despite the greater activity of both enzymes in whole cells after treatment with ethidium bromide for 5 days. This result suggests that when the replication and transcription of mitochondrial DNA is blocked completely by ethidium bromide, the synthesis of new mitochondrial membranes continues despite the lack of cell growth. Consequently, the amount of mitochondrial protein per cell has increased by nearly 50%. At present, electron micrograph studies are in progress to determine whether this increase in mitochondrial protein results from a greater number of mitochondria per cell or an increased size of individual mitochondria. Several earlier reports (6, 9) have indicated that treatment of mammalian cells with ethidium bromide for several days results in changes in mitochondrial structure especially in the appearance of the cristae.

Similar increases in the activity of different mitochondrial enzymes have been reported in Neurospora treated with either chloramphenicol or ethidium bromide. For example, the cyanide-insensitive alternate oxidase increases significantly when chloramphenicol is added to cultures (25), while the rifampicin-sensitive mitochondrial RNA polymerase plus certain elongation factors are drastically increased in Neurospora treated with either inhibitors of mitochondrial transcription or translation (26, 27). To explain these increases, Barath and Kintzel (26, 27) have suggested that mitochondrial DNA codes for a repressor pro-
tein(s) which is (are) synthesized in the mitochondria. These repressor proteins would then leave the mitochondria and be transported to the cell nucleus where they would act as repressors on nuclear genes. Treatment with chloramphenicol or ethidium bromide would inhibit the formation of these repressor proteins so that synthesis of mitochondrial proteins in the cytoplasm would no longer be regulated resulting in a continued increase in the amount of certain proteins.

An alternative explanation might be that the drop in total cellular ATP levels which must occur when the activity of the respiratory chain has been severely reduced by treatment with ethidium bromide or chloramphenicol may act to trigger some control mechanism which stimulates the synthesis of more mitochondrial membrane protein in an attempt to remedy the intracellular lack of ATP. It should be noted that a prime target of all of these inhibitors is cytochrome oxidase, the terminal step in the respiratory chain. Similarly, King et al. (7) observed that addition of appropriate concentrations of cyanide to cultures of L cells causes a decrease in growth and lowered cytochrome oxidase activity with concomitant increase in cytochrome c. They suggested that a lack of ATP might act as a stimulus for an increase in all mitochondrial constituents. It may not be necessary to suggest the presence of repressor-like proteins made in the mitochondria, since other possible metabolic controls have not been explored.

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