EFFECTS OF ETHIDIUM BROMIDE ON VARIOUS SEGMENTS OF THE RESPIRATORY CHAIN IN THE CELLULAR SLIME MOLD, *DICTYOSTELIUM DISCOIDEUM*

Robert N. STUCHELL, Bernard I. WEINSTEIN and Diana S. BEATTIE
Department of Biochemistry, Mount Sinai School of Medicine, 10 East 102nd St. New York, N.Y. 10029, U.S.A.

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1. Introduction

Ethidium bromide (EB) and other dyes, such as the acridines, which intercalate between the bases of DNA [1] selectively inhibit the replication and transcription of mitochondrial DNA [2, 3]. The early studies of Slonimski [4] demonstrated that the addition of EB to growing cultures of yeast caused the quantitative and irreversible conversion of wild-type cells to the cytoplasmic 'petite' mutation. Phenotypically, this mutation is characterized by the complete loss of mitochondrial cytochromes and consequently an absence of respiratory activity. In contrast, addition of EB to mammalian cells in tissue culture produces changes which are reversible. Treatment of human fibroblast cultures [5], L cells [6, 7], and SV 40-transformed cells [8] with EB for several days resulted in a drastic reduction in the content of cytochromes a–a_3 and a variable decrease in the level of cytochrome b. Furthermore, the amount of cytochromes c and c_1 in L cells increased nearly 2-fold after addition of EB to the culture medium [6, 7]. Morphologically, changes in mitochondrial structure including a reduction in cristae have been observed in EB-treated L cells [6]. However, in all the mammalian cells examined the effects of EB treatment were completely reversible.

The cellular slime mold *Dictyostelium discoideum* may prove to be 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 adequate nutrients. Under appropriate conditions, the amoebae aggregate into a multicellular mass and differentiate into more than one distinct cell type. It should be noted that this cellular differentiation occurs in the absence of any further cell division.

The effects of EB on mitochondrial formation and differentiation in the slime mold have been investigated. After addition of EB to cultures of slime mold amoebae, growth continued for only 1 or 2 more generations. After 1.5 generations in EB, a 50% decrease in the specific activity of cytochrome oxidase was observed, while a more gradual decrease in succinate–cytochrome c reductase activity occurred during the next five days in EB. In contrast, the specific activity of two membrane-bound enzymes, succinate dehydrogenase and NADH dehydrogenase, increased nearly 2-fold in cultures which had been treated with EB for five days. The viability and the capacity to differentiate was unchanged in cells treated with EB for five days.

2. Materials and methods

Dictyostelium discoideum (Strain Ax-3 from W.F. Loomis, Jr., University of California, San Diego) was grown in an axenic medium [9] containing 0.2 mg/ml of streptomycin sulfate. Solutions containing EB were freshly prepared, sterilized by filtration through a Millipore filter (0.22 μ) and added to the cultures to a final concentration of 10 μg/ml. The cells were grown in 100 ml of medium in 250 ml Erlenmeyer flasks at 23°C on a rotary shaker at 200 rpm. To prevent breakdown of the light-sensitive EB, cultures were maintained in the dark. Cells were harvested by centri-

fugation at 600 *g* for 10 min, washed two times in 0.01 M phosphate buffer, pH 7.0, and finally resuspended in phosphate buffer at a concentration of 1×10^8 cells/ml, (10–15 mg protein/ml). The cells were lysed by addition of Triton X-100 to a final concentration of 0.4%. This concentration of Triton was sufficient to cause 100% cell breakage without inhibiting any of the mitochondrial enzymes studied.

Cytochrome oxidase, succinate dehydrogenase, NADH dehydrogenase and succinate–cytochrome *c* reductase were assayed as described by Kim and Beattie [10] at room temperature in a Gilford recording spectrophotometer.

Adsorption spectra were obtained at room temperature on a Cary 14 recording spectrophotometer using cells lysed with Triton. The contents of the reference cuvette were oxidized with 0.1 mM Dicoumarol and a few grains of ammonium persulfate, while reduction was achieved with a few grains of sodium dithionite.

3. Results

Slime mold amoebae grow in liquid medium at 23°C with a generation time of 18 hr reaching stationary phase at a cell concentration of 1.2×10^7 per ml (fig. 1). After addition of 10 μg EB/ml, indicated by the arrow in fig. 1, the cells continued to grow at the normal rate for 1 to 2 generations. Subsequently, no further increase or decrease in cell number was observed in the EB-treated culture during the five days studied. Furthermore, the cells proved competent to recover from the EB treatment and resumed the normal growth rate after removal of the EB from the medium.

Despite the normal growth of the cells during the first day in EB, the specific activity of cytochrome oxidase in the cells decreased by more than 50% indicating the total cytochrome oxidase activity per culture remained nearly constant (fig. 2). Activity of this enzyme continued to decrease during the next four days in EB, reaching a level 40% of the control cells. In contrast, the specific activity of succinate–cytochrome *c* reductase also decreased in the cells treated with EB but at a much slower rate. During the first day in EB, the enzymic activity decreased less than 10%, but during the next four days the activity steadily decreased reaching a level of 65% of the control cells.

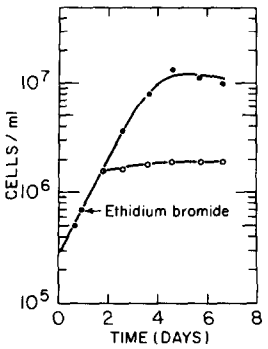

Fig. 1. Growth curve of control (●—●—●) and EB-treated (○—○—○) cells. Arrow indicates the time of EB addition.

The spectral traces (fig. 3) confirm the changes in enzymic activity of these two segments of the respiratory chain. The characteristic bands of cytochromes a–a_3, b, and c were observed at 605, 592 and 550 nm respectively in the control cells. After three days in EB (middle trace) the content of cytochrome a–a_3 and b have both decreased significantly compared to the control cells, while that of cytochrome c remained unchanged or increased slightly. The cytochrome

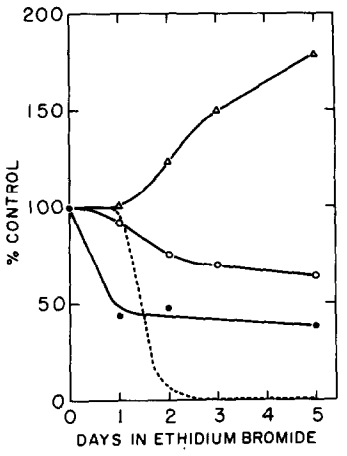

Fig. 2. The change in activity of mitochondrial enzymes during growth of cells in EB expressed in terms of per cent of control. Succinate dehydrogenase (△—△—△), control value 34.3 nmoles of substrate oxidized/min/mg of protein; succinate–cytochrome *c* reductase (○—○—○), control value 74.7 nmoles cytochrome *c* reduced/min/mg of protein; cytochrome oxidase (●—●—●) control value is the first order rate constant (k)/min/mg protein. The rate of growth (– – – –) compared to the control value of 18 hr per generation.

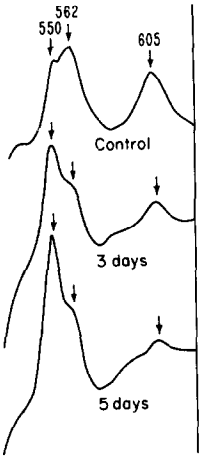

Fig. 3. The reduced–oxidized absorption spectra of the cytochromes of control and EB-treated cells as described in the test. Top trace – control cells, middle trace – cells treated with EB for three days, bottom trace – cells treated with EB for five days. Protein concentration was 13.5 mg/ml.

a–a_3 band was decreased even more after two additional days in EB (bottom trace). However, the absorbance at 562 and 550 nm due to cytochromes b and c showed a more complex response to the continued presence of EB. The peak at 562 nm representing cytochrome b appeared to be only slightly lower than the controls at this time, while the peak at 550 nm representing cytochrome c was 1.65-fold in excess of that present in the control cells.

Two other membrane-bound enzymes of the respiratory chain, succinate and NADH dehydrogenases, were also assayed in cells treated with EB. Succinate dehydrogenase activity remained constant during the first day in EB while the cells were continuing to divide (fig. 2). However, when cell growth had ceased in the EB-treated culture, the specific activity of succinate dehydrogenase showed a corresponding increase reaching a maximum 180% greater than that of the control cells after five days. The specific activity of NADH dehydrogenase was 165% greater than that of the control cells after five days in EB.

Cells which had been in EB-containing medium for five days were tested for their ability to differentiate when washed free of nutrients and placed on buffer-saturated pads as described by Sussman [11]. The development of spores and stalks appeared to proceed in a normal manner, although there were some atypical cell masses present. The spores when inoculated into fresh medium developed normally into free-living amoebae and retained sensitivity to subsequent addition of EB.

4. Discussion

In a previous study, Firtel and Bonner [12] demonstrated that EB inhibited any new synthesis of mitochondrial DNA in cultures of slime mold amoebae and caused a reduction of the total quantity of mitochondrial DNA in the cell. Our results indicate that addition of EB to slime mold cultures immediately blocks the formation of cytochromes a–a_3, determined either spectrophotometrically or by assay of cytochrome oxidase activity in the cells. Despite the consequent lowered activity of the respiratory chain, the cells continued to grow for one or possibly two generations. Presumably, when energy production by the respiratory chain had become limiting, the cells lost the ability to grow and divide. The synthesis of cytochrome b, determined spectrophotometrically or by the assay of succinate–cytochrome c reductase activity, was also inhibited by EB treatment but at a much slower rate than cytochromes a–a_3. Previous studies with yeast [13] and mammalian cells in tissue culture [6–8] have suggested that EB causes a primary effect on the formation of cytochromes a–a_3 and a slower, perhaps secondary, effect on the formation of cytochrome b.

In contrast, the total content of cytochrome c was increased nearly 2-fold in cells treated with EB for five days. The presence of multiple forms of cytochrome b in the cell [14] hinders the determination of the respiratory chain cytochromes b, c_1, and c. Hence, we are unable to distinguish cytochrome c_1 in whole cells and observe the effects of EB on its synthesis. Likewise, the increased absorption at 562 nm observed in cells grown in EB for five days compared to the cells in EB for three days may be due to an increase in another b-type cytochrome as described by Sato et al. [8]. Currently, spectrophotometric studies of isolated mitochondria obtained from both control and EB-treated cells are under investigation to clarify this point.

Of major interest is the increase in specific activity

of two membrane-bound flavoproteins of the mitochondrial respiratory chain in cells treated with EB. After growth of the cells had ceased,, the specific activity, and hence total activity, of both succinate and NADH dehydrogenase continued to increase during the five days studied. In a similar study, Barath and Küntzel [15] reported that addition of inhibitors of mitochondrial transcription and translation to cultures of *Neurospora* stimulated the synthesis of certain mitochondrial enzymes in the cytoplasm suggesting that mitochondrial gene products might act as repressors. Indeed, formation of both succinate and NADH dehydrogenases has been shown to require proteins synthesized only on cycloheximide-sensitive cytoplasmic ribosomes [10, 16].

Although growth of the slime mold stopped after one or two generations in EB, presumably due to the diminished energy production by the respiratory chain, these cells retained the ability to differentiate. These results suggest that the energy requirements for cellular differentiation may be less than those required for cell growth and division. In this context, Takeuchi [17] reported significant qualitative changes in different segments of the respiratory chain in slime mold cells undergoing the transition from the amoeboid to the multicellular stages. Possibly, a decrease in the intracellular energy level may be acts as control for the initiation of cellular differentiation.

Acknowledgements

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