ETHIDIUM BROMIDE AS AN UNCOUPLER OF OXIDATIVE PHOSPHORYLATION

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1. Introduction

The literature describing the biophysical and biological effects of ethidium bromide has been increasing rapidly [1–6]. Human fibroblast cultures [7] and L cells [8] exposed to ethidium bromide showed a reduction or loss of cytochromes aa_3 content. Sato et al. [9] demonstrated that during 1 to 8 days of ethidium bromide treatment on SV-40 virus transformed cell line not only cytochromes aa_3 but also cytochrome b and c_1 reduce in their amounts. The antitumor effect of ethidium bromide and of 14 of its newly synthesized related substances has been recently investigated [10].

However, less is known about the effects of the dye on some energy-producing processes in mitochondria. This report describes the effects of ethidium bromide on endogeneous respiration of ascites tumor cells as well as on mammalian mitochondrial respiration and ATPase activity.

2. Materials and methods

2.1. *Ascites tumor cells*

ELD (Ehrlich-Lettré hyperdiploid) ascites tumor cells were harvested 6–8 days after inoculation in ICR albino mice (0.2 ml ascites fluid) washed in a saline phosphate medium [11] and suspended in the same medium as described in earlier papers [12,13].

2.2. *Isolation of mitochondria*

Intact pigeon heart and rat liver mitochondria were isolated according to the method of Chance and Hagihara [14]. The protein concentration was determined by the biuret method [15].

2.3. *Measuring of respiration and ATPase activity*

Oxygen uptake was measured with a Clark type oxygen electrode as described previously [13]. All mitochondrial preparations were checked for structural integrity using the criterion of respiratory control [16]. Latent mitochondrial ATPase activity was measured by a pH electrode in 0.12 M KCl and 0.02 M Tris medium [17]. The ATPase hydrolysis rate was calculated assuming 0.8 H^+ ion was released per ATP molecule hydrolyzed [18].

2.4. *Chemicals and reagents*

Ethidium bromide was obtained from Dr Florence R. White, National Institutes of Health Bethesda and was dissolved in dimethyl sulphoxide (DMSO) final concentration 1 per cent [19]. FCCP carbonyl cyanide p-trifluoromethoxyphenylhydrazone was kindly supplied by Dr Heytler of E. I. DuPont de Nemours Co. All other reagents were obtained from Sigma Chemical Co.

3. Results

As can be seen from fig.1 ethidium bromide inhibited endogeneous respiration of ELD cells in concentration dependence (left). Maximal inhibition of respiration (30%) was reached at the concentration of ethidium bromide 0.5 to 1 mM. Inhibited respiration

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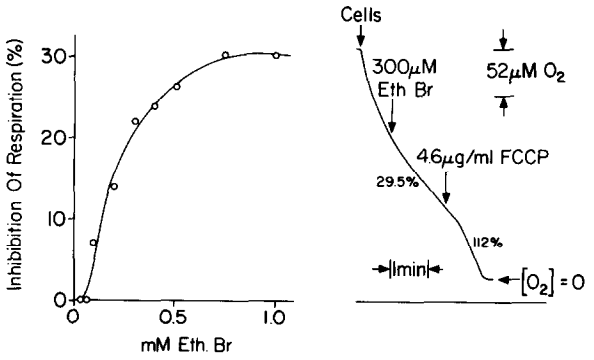

Fig.1. Effect of ethidium bromide on endogenous respiration of ELD cells (left) and reversal of this inhibition by FCCP. 0.2 ml of cell suspension, containing 17.5 mg dry weight cells, were added to 2.0 ml of isotonic saline–phosphate medium pH 7.4 (for composition see Materials and methods). Oxygen uptake was measured at 30°C. Rate of oxygen consumption by the samples with 0 ethidium bromide was 135 nmol/min.

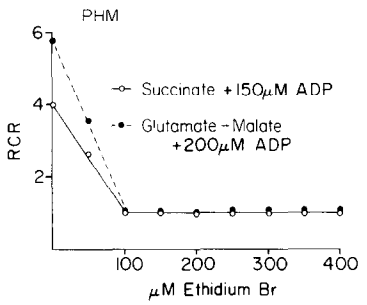

Fig.3. Effect of ethidium bromide on respiratory control ratio (RCR) of pigeon heart mitochondria. The other conditions were the same as for fig.2. State 3 respiration with succinate as a substrate was measured in the presence 150 μM ADP and 200 μM ADP in the presence of glutamate plus malate as shown in figure respectively.

can be released by FCCP. Fig.2 shows stimulation of oxygen uptake in pigeon heart mitochondria in the presence of various concentrations of ethidium bromide. Similar results were obtained with rat liver mitochondria

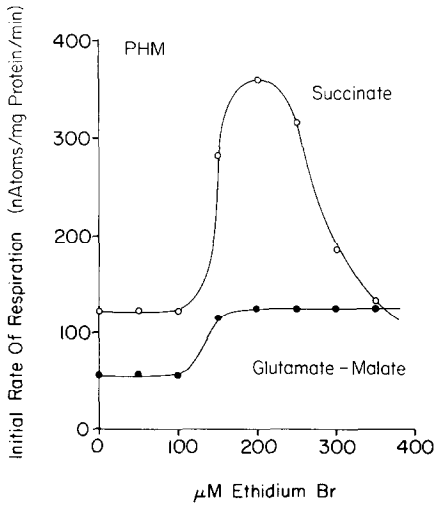

Fig.2. Effect of ethidium bromide on rate of oxygen uptake of intact pigeon heart mitochondria (PHM). The mitochondria were suspended at 0.6 mg protein/ml in a medium containing 0.225 M mannitol, 0.075 M sucrose, 10 mM K_2HPO_4 and 10 mM MOPS pH 7.2. The substrates were 10 mM succinate in the presence of 3 μM rotenone or 5 mM glutamate plus 5 mM malate. The reaction temperature was 25°C.

but stimulation of respiration in state 4 is lower and higher concentrations of dye were needed. Up to 100 μM there is no stimulation of oxygen uptake in the presence of either succinate or glutamate plus malate as substrates. Maximal stimulation of respiration in the presence of ethidium bromide was obtained at a concentration of 200 μM. If the substrate was succinate with increasing concentrations of ethidium bromide there was a decrease in oxygen uptake. Excessive concentrations of uncouplers of oxidative phosphorylation inhibit both mitochondrial respiration and the uncoupler-induced ATPase activity [20–24]. Ethidium bromide decreased the respiratory control ratio very sharply which suggests that ethidium bromide is a potent uncoupling agent (fig.3). The typical polarographic traces showing the oxidation of succinate and/or glutamate plus malate by pigeon heart mitochondria in the presence of ethidium bromide are shown in figs.4 and 5 respectively. After addition of pigeon heart mitochondria to the medium containing succinate plus 150 μM ethidium bromide first, there is no stimulation of respiration (109 natoms of oxygen/mg protein/min as for control experiments). The stimulation starts about 2 min later and the respiration rate remains linear (the maximal rate of respiration was calculated from this part of the curve). The pigeon heart mitochondria were uncoupled in the presence of ethidium bromide.

Similar experiments were carried out with NAD-

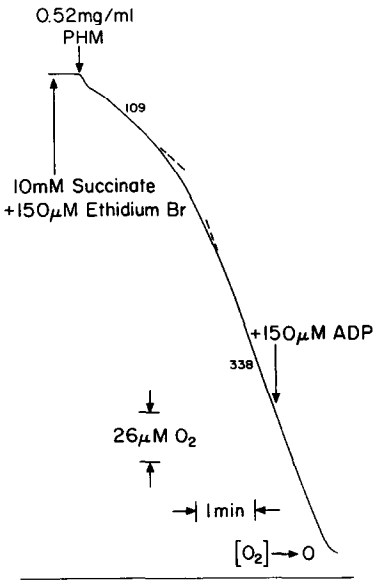

Fig.4. Typical time-course polarographic trace showing oxidation of succinate in the presence of 150 μM EB by pigeon heart mitochondria. The figures on the lines represent the rates of oxygen uptake in natoms/mg/min. The other conditions were the same as for fig.2.

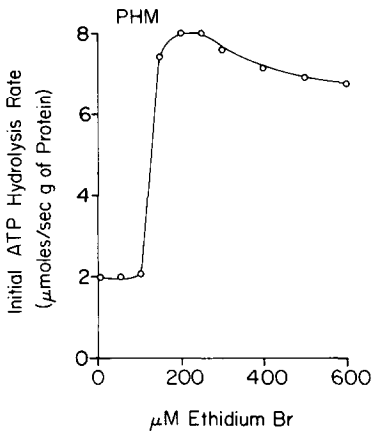

Fig.6. Effect of ethidium bromide on activation of mitochondrial ATPase activity. Pigeon heart mitochondria (0.38 mg protein/ml) were suspended to a final vol of 4.0 ml in a 0.12 M KCl and 0.02 M Tris–Cl medium (pH 7.1). The ATP was added to a final concentration of 1.0 mM and the rate of ATP hydrolysis was measured with a sensitive recording pH meter (10 mV full scale) by the method of Nishimura et al. as described in Materials and methods. The reaction was started by adding pigeon heart mitochondria (PHM). Mitochondria were incubated at 25°C. Control experiments gave an initial rate of hydrolysis of 2.0 μmol ATP per second per gram of protein.

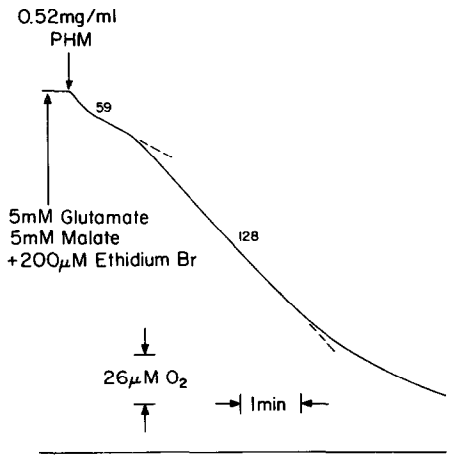

Fig.5. Polarographic trace showing effect of EB on the oxidation of glutamate plus malate by pigeon heart mitochondria. The other conditions were the same as for fig.2. The figures on the line represent the rate of oxygen uptake in natoms/mg protein/min.

linked substrates (fig.5). As we have indicated elsewhere in a brief report [25], a more direct measure of uncoupling properties of ethidium bromide is seen in fig.6 where the effect on the initial rate of ATP hydrolysis is shown. In a similar way in fig.2 a latent mitochondrial ATPase activity is stimulated only by concentrations of ethidium bromide higher than 100 μM. Maximal stimulation of ATPase activity occurs at 200 μM of ethidium bromide. The same concentrations of ethidium bromide were needed for maximal stimulation of respiration (see fig.2). Fig.7 shows traces from measuring ATPase activity in the presence of different concentrations of ethidium bromide. With increasing concentrations of ethidium bromide inhibition of ATPase activity began to appear (traces A, B and C). In agreement with the results of Kraayenhoff [26] higher concentrations of DNP have an inhibitory effect on ATPase activity. The figures on the traces represent the μmoles of ATP hydrolyzed per sec per g of protein. As shown in table 1, addition of ADP to pigeon heart mitochondria

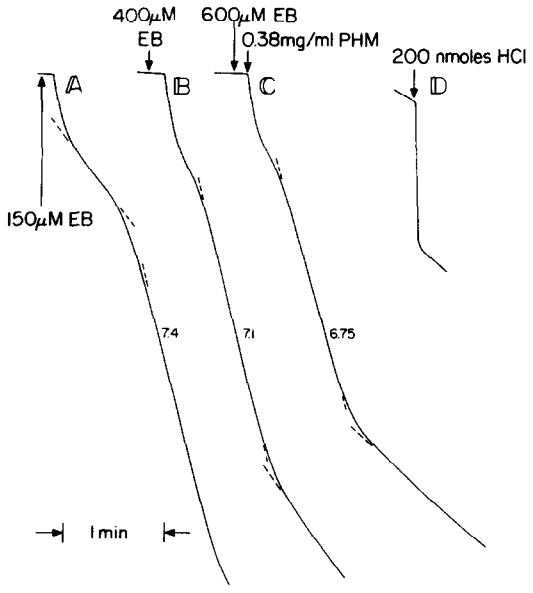

Fig.7. Effect of different concentrations of ethidium bromide on time-course of ATPase activation. The pigeon heart mitochondria (0.38 mg protein/ml) were suspended to a final vol of 4.0 ml. Other conditions were the same as for fig.6.

oxidizing glutamate plus malate produced an increase in the respiratory rate from 33.2 to 145.9 natoms of oxygen/mg protein per min. The respiratory rate decreased to 28.1 when oligomycin was added. After oligomycin treatment normal mitochondria became refractory to added ADP but ethidium bromide was capable of partially converting the inhibited mitochondria from the coupled state to an uncoupled condition. The observed reversal of oligomycin inhibition of respiration is further evidence of uncoupling activity of ethidium bromide.

Table 1
Inhibition by oligomycin of the ADP-stimulated oxidation of glutamate-malate by pigeon heart mitochondria and reversal of this inhibition by ethidium bromide

Rate of oxygen consumption (natoms/mg protein/min)				
State 4	+ADP	+Oligomycin	+Ethidium bromide added (μM)	Reversal of inhibition by ethidium bromide
33.2	145.9	28.1	0	28.1
31.2	146.0	27.7	50	27.7[a]
34.6	139.0	31.2	100	31.2
31.2	146.2	31.2	200	62.2
34.6	146.0	31.2	300	69.5
31.2	139.2	31.2	400	69.5
34.6	139.0	31.2	500	83.5
31.2	159.0	31.2	700	83.5
34.6	167.0	31.2	800	83.5
41.6	153.0	27.7	+0.1 μM FCCP	145.0

[a] Initial rate of oxygen consumption. The mitochondria were suspended at 0.75 mg protein/ml in a medium containing 0.225 M mannitol, 0.075 M sucrose, 10 mM K_2HPO_4, 0.2 mM EDTA and 10 mM MOPS, pH 7.2. The substrates were 5 mM glutamate and 5 mM malate. The reaction temperature was 25°C. Respiration was measured with no additions (state 4) and after sequential addition of 200 μM ADP (+ADP), 0.6 μg oligomycin/mg protein (+oligomycin) and the indicated concentration of ethidium bromide (+EB).

4. Discussion

Ethidium bromide inhibited state 3 of respiration, stimulated state 4 of respiration and lowered respiratory control ratio (RCR) when succinate and/or NAD-linked substrates were used. Energy production was inhibited,, as evident by the decrease in the RCR (fig.3). A typical uncoupler of oxidative phosphorylation increases the respiration rate proportionally to the concentrations used. In the case of ethidium bromide up to 100 μM there is no stimulation either of respiration or ATPase activity. After this concentration of ethidium bromide a rapid stimulation of both respiration and ATPase activity began to occur (figs.2 and 6).

Most uncoupling agents are weakly acidic and lipophilic and are presumed to exert their uncoupling effects through a non-specific interaction with mitochondrial membranes. Ethidium bromide is also bound strongly to mitochondrial membanes [27,28] and ATPase (F_1) is attached to M-site of the inner membrane [29,30]. As shown in fig.6 ATPase activity was also induced by addition of the dye of ethidium bromide. These results are in good agreement with those obtained by Miko and Drobnica (in preparation). 300 μM ethidium bromide decreased the level of ATP in rat liver mitochondria from 10.1 (control) to 8.7 and 600 μM to 6.8 m μmol of ATP per mg protein respectively. FCCP (2 μM) under the same conditions decreased the level of ATP to 1.9 m μmol ATP/mg protein. Krempasky et al. [31] found that in vivo administration of ethidium bromide did not affect the activity of neither the latent nor the uncoupler stimulated ATPase activity of the mitochondria regenerating liver. However results of this paper suggest a little activation of a latent ATPase activity (increase in ATPase activity from 9.8 in control to 14.9 and 15.5 nmol of P_i released respectively). Mahler and Perlman [32] found that ethidium bromide induced cells contain a measurable and significant amount of oligomycin sensitive ATPase (F_1).

From results presented we can conclude that ethidium bromide is similar to other uncouplers (such as DNP and FCCP) in that it: 1. stimulates respiration in state 4 mitochondria; 2. stimulates mitochondrial ATPase activity; 3. partially releases the inhibition of mitochondrial respiration by oligomycin and 4. at higher concentrations inhibits both mitochondrial respiration and mitochondrial ATPase activity.

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