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Ethidium Bromide Inhibits Mitochondrial Phosphorylating Oxidation (38957)

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Ethidium bromide (2,7-diamino-9-phenyl-phenanthridinium-10-ethyl bromide) inhibits mitochondrial DNA (1), RNA (2), and protein syntheses (3, 4). Ethidium bromide can intercalate with the nucleic acids (5) and it is commonly thought that inhibition of these processes occurs by this mechanism rather than by inhibiting ATP generation. Ethidium bromide can combine with mitochondrial membranes (6) producing a color shift in the ethidium spectrum which indicates interaction with a low dielectric site on the membrane. This interaction is enhanced by the presence of ATP but not ADP nor AMP (7). Degradation of mitochondrial DNA may imply that energy processes are inhibited by ethidium bromide and ethidium bromide was considered an uncoupler of oxidative phosphorylation (8).

Since an intact energy transducing membrane is required to show temperature discontinuities in Arrhenius plots for state 3 and state 4 respirations (9), we have studied the interaction of ethidium bromide on respiratory control in *intact* mitochondria. This communication presents evidence that ethidium bromide, at mutagenic concentrations of 1 μg/10^6 liver cells, is a phosphorylation inhibitor and not an uncoupler of mitochondrial respiratory control processes. Exhaustive washing of mitochondria did not remove inhibition caused by ethidium bromide nor did it effect more than 27% removal of bound dye.

Materials and Methods. Hepatic mitochondria were prepared from Sprague–Dawley rats as described previously (10). Respiratory rates were determined polarographically (10) and the conventions of Chance and Williams (11) were used to identify respiratory states and in computing respiratory control ratios (RCR). High amplitude volume changes of the organelles (swelling and contraction cycles) and mitochondrial ATPase (ATP phosphohydrolase, EC 3.6.1.4) activity were monitored and assayed as already described (12). Uncoupler stimulated ATPase and energized contraction of the organelles were both inhibited completely by oligomycin (3 and 6 μg/mg mitochondrial protein, respectively). Mitochondrial protein was solubilized with 1% deoxycholate and estimated by a biuret method (13). Sucrose, EDTA, and sodium deoxycholate were from Fisher Scientific Co.; mannitol was from Pfanstiehl Laboratories; ethidium bromide, ADP, ATP, substrates, and other biochemicals were from Sigma Chemical Co. Ethidium bromide solutions were protected from light at all times.

Results. Ethidium bromide depressed P_i acceptor control of respiration in mitochondria oxidizing either glutamate or succinate (Fig. 1). The effect was concentration dependent and resulted from selective inhibition of respiration in the active state (state 3). This inhibition was specifically responsible for complete depression of the RCR to unity; at no time were the organelles released from control by uncoupling, *i.e.*, the resting state respiration (state 4) was unaltered by ethidium bromide throughout its effective concentration range. As in the case of depression of the RCR by a large number of lipophilic organic cations (10), glutamate respiration was more sensitive to such agents than was succinate. The observations were in accord with the greater lipophilicity of the NADH dehydrogenase receptor site compared to the succinate dehydrogenase receptor site (10). In terms of its degree of depression of the RCR, a given concentration of ethidium bromide was about twice as effective on mitochondria oxidizing glutamate as on organelles oxidizing succinate.

Further evidence for eliminating the possibility that ethidium bromide might diminish

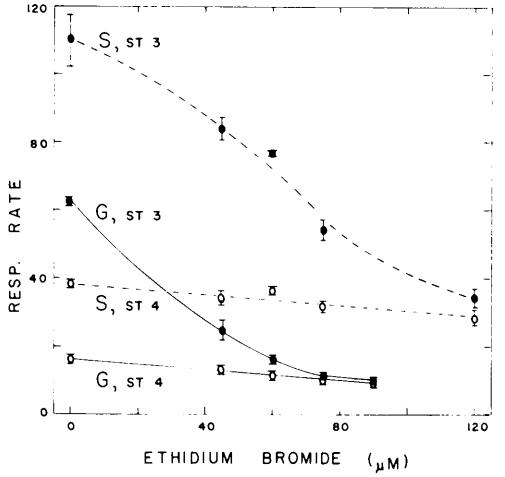

FIG. 1. Influence of ethidium bromide on respiratory states of mitochondria. Respiratory rates (ng atoms oxygen/min/mg mitochondrial protein) were assayed polarographically at 30° with a Clark fixed voltage electrode. The 3 ml reaction mixture (pH 7.4) contained 0.33 *M* mannitol, 3.5 m*M* potassium phosphate, 3.5 m*M* KCl, 0.33 m*M* EDTA, 4 mg dialyzed crystalline bovine serum albumin, 1.4 m*M* glutamate (——) or succinate (– – –), and mitochondria corresponding to 2.5 mg mitochondrial protein. RCR was computed as the ratio of the respiratory velocity in state 3 (●) after addition of 0.4 μmole ADP to the velocity in state 4 (○) after exhaustion of ADP. Mean of four mitochondrial preparations ± SEM.

TABLE I. HIGH AMPLITUDE VOLUME CHANGES OF MITOCHONDRIA[a]

Treatment	ΔA_{520}/mg mitochondrial protein × 10^3	
	Swelling	Contraction
Control	−19 ± 6	+50 ± 9
5 μ*M* sodium oleate	−128 ± 32	+130 ± 22
100 μ*M* ethidium bromide	−30 ± 12	+133 ± 13
5 μ*M* oleate + 100 μ*M* ethidium	−76 ± 22	+126 ± 36

[a] Swelling was recorded as negative absorbance changes at 520 nm until the tracing plateaued (2–3 min for spontaneous controls and ethidium bromide treated preparations; 5–6 min for sodium oleate treated preparations) using a Gilford recording spectrophotometer thermally controlled at 30°. Mitochondria (0.5 mg protein) were suspended in 125 m*M* KCl containing 20 m*M* Tris, pH 7.4. Contraction, initiated by 5 m*M* ATP, 3 m*M* $MgCl_2$, and 6 mg bovine serum albumin (3 ml final volume) was followed as the positive absorbance change to the new plateau (1–3 min). Mean ± SEM for four mitochondrial preparations.

the RCR by uncoupling respiration from phosphorylation was obtained by monitoring mitochondrial high amplitude volume changes (Table I). Although the mitochondria were subject to pronounced swelling by oleate, ethidium bromide had no significant influence on osmotic integrity and it did not hinder energized contraction of the organelles. Neither did it augment oleate swelling nor subsequent contraction energized by ATP. Calcium swelling was also unaffected by ethidium bromide. In addition, ethidium bromide did not release the latent ATPase of mitochondria (Table II) and it did not enhance uncoupler-stimulated mitochondrial ATPase activity. The ethidium bromide concentration used in these studies was sufficient to eliminate respiratory control by P_i acceptor (*cf.* Fig. 1).

Extent of reversibility of the ethidium bromide–mitochondrial interaction was investigated on the bases of restoration of functional activity and dye binding as a consequence of washing the organelles. Data in Table III indicate that repeated washing resulted in no restoration of state 3 respiration nor of the RCR and that the dye was bound irreversibly by mitochondria. This same technique reversed completely the depression of respiratory control produced by the lipophilic quaternary salt tetrabutylammonium bromide and by dinitrophenol (K. S. Rogers and E. S. Higgins, in preparation). After exhaustive washing (seven times) which led to some damage to the mitochondria, 73.2 ± 3.3% of the ethidium bromide remained associated with the organelles.

An attempt was made to remove ethidium bromide from the treated mitochondria by dialysis against a cation exchange resin. These results are presented in Table IV. Storage or dialysis of untreated mitochondria for 17 hr at 3 ± 1° resulted in some depression of both resting and active state respiratory velocities, but not in respiratory control (Expts 1, 2, and 3). Dialysis against the ion exchange resin under identical conditions resulted in marked respiratory depressions and reduction of the respiratory control

TABLE II. MITOCHONDRIAL ATPASE ACTIVITIES[a]

Treatment	nmoles $P_i \cdot min^{-1} \cdot mg\ protein^{-1}$
Spontaneous	67 ± 3
100 μM ethidium bromide	64 ± 6
100 μM 2,4-dinitrophenol	455 ± 15
100 μM ethidium + 100 μM dinitrophenol	528 ± 30

[a] Mitochondria (2 mg protein) were incubated at 30° for 5 min in 2 ml of a mixture (pH 7.4) of 150 mM sucrose, 15 mM Tris, and 5 mM ATP. Blanks were prepared without mitochondria and initial rate kinetics were maintained. P_i was determined according to Lowry (17). Mean ± SEM for five mitochondrial preparations.

TABLE III. DEPRESSION OF RESPIRATORY CONTROL BY ETHIDIUM BROMIDE AND FAILURE OF RESTORATION BY SERIAL WASHING[a]

Number of washes	State 3		RCR[b]		Percentage of dye bound
	Control	Ethidium	Control	Ethidium	
0	56.5 ±2.1	13.1 ±2.1	3.16 ±0.20	1.65 ±0.24	100
1	39.3 ±3.5	13.2 ±1.5	3.11 ±0.02	1.73 ±0.15	97.5 ±1.0
2	38.5 ±6.7	9.3 ±1.5	2.62 ±0.13	1.63 ±0.06	94.4 ±1.1
3	36.3 ±1.2	7.8 ±1.2	2.99 ±0.13	1.64 ±0.22	81.6 ±3.2

[a] Liver homogenates were divided in half and mitochondria were isolated from each portion. One pellet was suspended in isolation medium and the other in medium containing 0.2 μmole ethidium bromide/mg mitochondrial protein. Mitochondria were separated again by centrifugation and washed in isolation medium three times. An aliquot of each preparation (2.5 mg protein) was removed at each stage for assay of respiratory control and mitochondrial protein. Respiratory rates are in nanograms of atoms of oxygen per minute per milligrams of mitochondrial protein with glutamate as substrate. The fraction of the dye bound to mitochondria was estimated from the 486 nm absorbance of the supernate by difference. Untreated mitochondria served as controls. Mean ± SEM for three mitochondrial preparations.

[b] Respiratory control ratio.

ratio (RCR) by about 50% (Expt 4). However, complete inhibition of phosphorylating oxidation by ethidium bromide (Expt 5) could be overcome by dialysis for 17 hr against the resin (Expts 6 and 4). This treatment removed all but 3.5 ± 0.6% of the dye originally bound by mitochondria. Dialysis of ethidium bromide–mitochondrial preparations against the resin for shorter time intervals, *e.g.*, 4 hr, removed only about 75% of the bound dye.

TABLE IV. REMOVAL OF ETHIDIUM BROMIDE FROM TREATED MITOCHONDRIA[a]

Experiment	Conditions	Protein	State 3	RCR[b]
1	Zero time, control	2.6 ±0.1	71.5 ±9.7	3.5 ±0.1
2	Control	5.2 ±0.1	58.5 ±2.1	4.2 ±0.2
3	Dialyzed	8.3 ±1.1	41.1 ±3.2	3.2 ±0.3
4	Dialyzed *vs* resin	10.8 ±1.4	5.3 ±0.8	1.9 ±0.1
5	Zero time, Ethidium bromide	5.3 ±0.1	7.5 ±0.3	1.0 ±0
6	Ethidium bromide dialyzed *vs* resin	7.6 ±0.7	5.9 ±1.2	1.8 ±0.1

[a] Liver homogenates were divided in half and mitochondria were isolated from each portion. One pellet was suspended in isolation medium and the other in medium containing 0.2 μmole ethidium bromide/mg mitochondrial protein. The suspension without ethidium bromide was divided into three portions. One portion was kept on ice (Expt 2), the second portion was dialyzed against 200 ml of 0.25 M sucrose and 0.5 mM EDTA, pH 7.4 (Expt 3), and the third portion was dialyzed against an identical solution plus 10 g Amberlite C G-50 type I cation exchange resin (Fisher Sci. Co.) (Expt 4). Prior to use the resin was washed with 1% NaOH, distilled water, 1% HCl, and then adjusted to pH 7.4 with NaOH. Ethidium bromide treated mitochondria were dialyzed against resin in the same fashion (Expt 6). Each of the treatments (with the exceptions of Expt 1 and 5) was for 17 hr at 3 ± 1°. *Protein* represents milligrams of mitochondrial protein employed in the respiratory assays. Respiratory rates are in nanograms of atoms of oxygen per minute per milligrams of mitochondrial protein with glutamate as substrate. Mean ± SEM for three mitochondrial preparations.

[b] Respiratory control ratio.

Discussion. The concentration of ethidium bromide required for effective inhibition of ADP induced mitochondrial respiration is 100 μM. This corresponds to 2.95 mg of acridine dye for the mitochondria obtained

from 2 g of rat liver. Since 1 g of rat liver contains 4.39 mg of DNA (14) and assuming that the DNA content per cell is about 3×10^{-6} μg (15), then approximately 1 μg of ethidium bromide was added to the mitochondria from 10^6 rat liver cells. Thus the concentration which was effective as a mutagen and metabolic inhibitor (1–6) is also effective in disrupting energy metabolism. Therefore the ability of ethidium bromide to inhibit metabolic processes may be due in part to depressing synthesis of ATP (16) from mitochondrial oxidation of substrates.

Summary. Ethidium bromide, in addition to combination with mitochondrial nucleic acids, is a phosphorylation inhibitor during glutamate and succinate respiration by mitochondria. Exhaustive washing of ethidium bromide-treated mitochondria did not relieve the inhibition nor significantly decrease the amount of bound dye. Dialysis against a cation exchange resin at 3° for 17 hr removed about 97% of bound dye. This restored phosphorylating capacity to that of untreated mitochondria which had also been dialyzed against the resin. Since state 3 respiration was diminished and state 4 was unaffected by the presence of the acridine dye, and since neither swelling of mitochondria nor release of latent ATPase was observed, then ethidium bromide was not an electron transport inhibitor nor an uncoupler of oxidative phosphorylation. Inhibition of metabolic processes by ethidium bromide may be due in part to depressed generation of mitochondrial ATP.

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1. Mattoccia, L. P., and Roberti, S., *Biochem. Biophys. Res. Commun.* **60,** 882 (1974).
2. Zylber, E., Vesco, C., and Penman, S., *J. Mol. Biol.* **44,** 195 (1969).
3. Lederman, M., and Attardi, G., *J. Mol. Biol.* **78,** 275 (1973).
4. Grivell, L. A., and Mitz, V., *Biochem. Biophys. Res. Commun.* **55,** 125 (1973).
5. Von Hippel, P. H., and McGhee, J. D., *Ann. Rev. Biochem.* **41,** 231 (1972).
6. Gitler, C., Rubalcava, B., and Caswell, A., *Biochim. Biophys. Acta* **193,** 479 (1969).
7. Azzi, A., and Santato, M., *Biochem. Biophys. Res. Commun.* **44,** 211 (1971).
8. Bastos, R. N., and Mahler, H. R., *J. Biol. Chem.* **249,** 6617 (1974).
9. Lee, M. P., and Gear, A. R. L., *J. Biol. Chem.* **249,** 7541 (1974).
10. Rogers, K. S., and Higgins, E. S., *J. Biol. Chem.* **248,** 7142 (1973).
11. Chance, B., and Williams, G. R., *Advan. Enzymol.* **17,** 65 (1956).
12. Higgins, E. S., and Banks, W. L., *Biochem. Pharmacol.* **20,** 1513 (1971).
13. Gornall, A. G., Bardawill, C. J., and David, M., *J. Biol. Chem.* **117,** 751 (1949).
14. Banks, W. L., Kline, E. S., and Higgins, E. S., *J. Nutr.* **100,** 581 (1970).
15. Boivin, A., Vendrely, R., and Vendrely, C., *C. R. Acad. Sci.* **226,** 1061 (1948).
16. Mitra, R. S., and Bernstein, I. A., *Arch. Biochem. Biophys.* **141,** 519 (1970).
17. Lowry, O. H., "Methods in Enzymology," Vol. 4, p. 366. Academic Press, New York (1957).

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