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SIDEDNESS OF INHIBITION OF ENERGY TRANSDUCTION IN OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA BY ETHIDIUM BROMIDE *

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Summary

Ethidium bromide, a new type of inhibitor of energy transduction in oxidative phosphorylation, inhibited ATP synthesis in intact mitochondria but not in submitochondrial particles, the latter being inside-out relative to the membranes of intact mitochondria. Ethidium bromide incorporated inside the submitochondrial particles inhibited ATP synthesis in the particles. The decrease of the membrane potential by valinomycin (plus KCl) inhibited only slightly the energy-dependent binding of ethidium bromide to the mitochondria.

The present results show clearly that ethidium bromide inhibited energy transduction in oxidative phosphorylation by acting on the outer side (C-side) of the inner mitochondrial membrane, perhaps by neutralizing negative charges created on the surface of the C-side, and that it had no inhibitory activity on the inner side (M-side) of the membrane. The present results show also that the energy-dependent binding of ethidium is not due to electrophoretic transport down the membrane potential; ethidium may bind to negative charges on the surface of the C-side. The present study suggest that an anisotropic distribution of electric charge in the inner mitochondrial membrane is an intermediary high energy state of oxidative phosphorylation.

Introduction

Fluorescence dyes with positive and negative charges have opposite effects on energized membranes of mitochondria and their effects on mitochondria

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

and submitochondrial particles are also opposite [1—3]: energization of intact mitochondria, induced with respiratory substrates or ATP, increases the binding of positively-charged fluorescent dyes (e.g., ethidium bromide [2—5]) to the membranes, but decreases the binding of negatively charged dyes (e.g., anilinonaphthalene sulfonate [1,2,6—8]). In contrast, energization of submitochondrial particles, which are inside-out relative to the membranes of intact mitochondria [9—21], has the opposite effects on dye binding.

Skulachev et al. [14,22—24] explained this sidedness of the interaction between fluorescent dyes and the membrane as being a result of electrophoretic transport of ions through the membrane down the membrane potential generated by respiration or ATP hydrolysis, in the scheme of Mitchell [25].

On the basis of the sidedness, Azzi [1,2] proposed that in the energized state the outer side (C-side) of inner mitochondrial membrane is more negative than the medium while the inner side (M-side) is more positive than the matrix (see Fig. 3 in ref. 1).

In studies on the problem of sidedness, we examined the sidedness of inhibition of energy transduction in oxidative phosphorylation by ethidium bromide. A preliminary account of this work has appeared [48].

Materials and Methods

Ethidium bromide and ADP were purchased from Aldrich Chemical Co., Milwaukee (U.S.A.) and Oriental Yeast Co., Osaka (Japan), respectively. ATP and antimycin A were products of Kyowa Hakko Kogyo Co., Tokyo. Valinomycin, CCCP and hexokinase (type III) were obtained from Sigma Chemical Co., St. Louis, Miss. $^{32}P_i$ was a product of Japan Radioisotope Association, Tokyo (Japan), and was purified by the method of Suelter et al. [26].

Rat liver mitochondria were isolated by the method of Hogeboom [27], as described by Myers and Slater [28], except that 0.25 M sucrose containing 2 mM Tris (pH 7.4) was used for homogenization and two washings [29]. Submitochondrial particles were prepared by a modification of the method of Hansen and Smith [30] as follows. Rat liver mitochondria were suspended at a concentration of 40 mg protein per ml in medium (pH 7.4) containing 0.25 M sucrose/10 mM Tris/1 mM ATP/1 mM $MgCl_2$/1 mM $MnCl_2$/1 mM potassium succinate and maintained at −15°C overnight. The mitochondrial suspension was thawed just before preparation of the particles and centrifuged at 18 000 × *g* for 10 min. The resulting mitochondria were suspended in 10 mM sodium pyrophosphate (pH 7.4) at a concentration of 20 mg protein per ml and treated in a Kubota Model 200M Insonater at 160 W (9 KHz) for 1 min at about 2°C. The resulting suspension was centrifuged at 25 000 × *g* for 20 min and the supernatant was recentrifuged at 144 000 × *g* for 30 min. The final pellet was suspended in 0.25 M sucrose (containing 2 mM Tris) and used as the preparation of submitochondrial particles.

To measure binding of ethidium to mitochondria, the mitochondria (1 mg protein/ml) were incubated for 5 min with a known concentration of ethidium bromide in the presence of 0.36 μg of rotenone/10 mM succinate/5 mM $MgCl_2$/2 mM EDTA/15 mM KCl/25 mM Tris/50 mM sucrose at pH 7.4. The incuba-

tion mixture was shaken gently during the reaction. Then the mixture was rapidly cooled to approx. 0°C and centrifuged at 8000 × *g* for 2 min in an Eppendorf Model 3200 microcentrifuge, and the remaining dye was estimated by measuring either its absorbance at 480 nm with a Hitachi Model 556 two-wavelength, double-beam spectrophotometer or its fluorescence at 610 nm using an excitation wavelength of 485 nm with a Hitachi Model MPF 3 spctro-fluorometer. In the latter case, bovine serum albumin (6.7 mg protein/ml) was added to increase the fluorescence of the dye.

Protein was estimated from the contents of cytochromes $a + a_3$ in intact mitochondria and submitochondrial particles as described previously [29,31], unless otherwise stated.

The amount of $^{32}P_i$-labeled substances was determined by the method of Nielsen and Lehninger [32] as modified by Avron [33].

Results

Asymmetry of the mitochondrial inner membrane with respect to inhibition of energy transduction by ethidium bromide

In the present paper we assumed the anisotropic electric charge distribution in the membrane proposed by Azzi [1,2] to be an intermediary high energy state on the main pathway of oxidative phosphorylation. If this assumption were valid, the energy-dependent binding of amphipathic cations to the surface of the C-side of energized intact mitochondria should neutralize the negative electric charge on this surface, resulting in inhibition of energy transduction in oxidative phosphorylation. Similarly, the energy-dependent binding of amphipathic anions to the surface of the M-side of energized mitochondria should neutralize the positive electric charge on the latter, and this also should inhibit energy transduction in oxidative phosphorylation.

If these ions penetrate the inner mitochondrial membrane very slowly, addition of amphipathic cations to intact mitochondria should inhibit oxidative phosphorylation in the membrane, whereas their addition to submitochondrial particles should not be inhibitory, because the membranes of the latter have the opposite orientation. Conversely amphipathic anions should inhibit oxidative phosphorylation in submitochondrial particles but not mitochondria.

Fig. 1 shows that 100 μM ethidium bromide (amphipathic cation) strongly inhibited the increased rate of oxygen uptake with a phosphate acceptor in intact mitochondria with succinate as substrate. This inhibition was released by addition of the uncoupler 2,4-dinitrophenol (Fig. 1). The inhibition was maximal approx. 3 min after addition of the dye to the mitochondrial suspension in the presence of succinate. Ethidium bromide had similar effects with glutamate and malate as substrate. Thus, when added to intact mitochondria it seems to inhibit energy transduction in oxidative phosphorylation, irrespective of the substrate. Fig. 2 shows that concentrations of up to 200 μM ethidium bromide inhibited ADP-stimulated respiration (state 3) in intact mitochondria, but did not affect state 4 respiration in the membranes; at concentrations of above 200 μM it stimulated state 4 respiration in the membranes, as reported by Miko and Chance [34]. This stimulation was dependent on the presence of inorganic phosphate (Fig. 2).

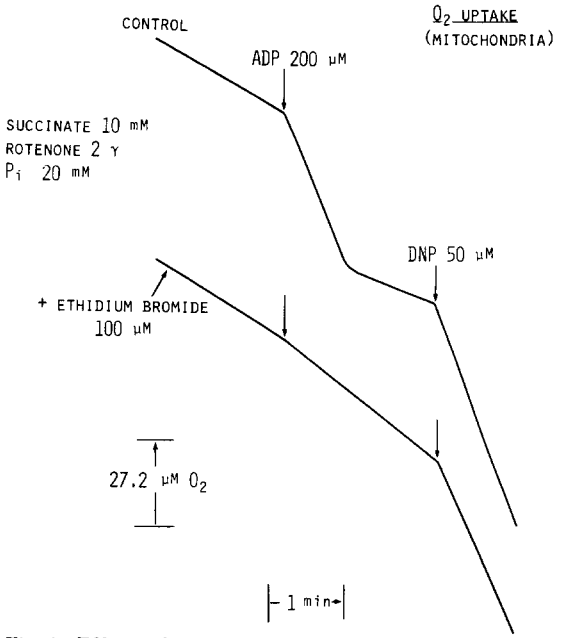

Fig. 1. Effect of ethidium bromide on oxygen uptake by intact mitochondria. Intact mitochondria (1 mg protein/ml) were preincubated for 5 min at 25°C in the presence of 10 mM succinate, 2 μg of rotenone, 20 mM potassium phosphate, 5 mM $MgCl_2$, 2 mM EDTA, 15 mM KCl, 50 mM sucrose, 25 mM Tris and 100 μM ethidium bromide in a final volume of 4.5 ml at pH 7.4. ADP and DNP (2,4-dinitrophenol) were added as indicated. Oxygen uptake was measured polarographically with a Yellow Spring Model YSI-53 Oxygen Monitor.

Fig. 3 shows that ethidium bromide greatly inhibited ATP-P_i exchange in intact mitochondria and Fig. 4 shows that 200 μM ethidium bromide completely inhibited ATP synthesis in intact mitochondria. However, Fig. 4 also shows that concentrations of up to 1.5 mM ethidium bromide had no affect on

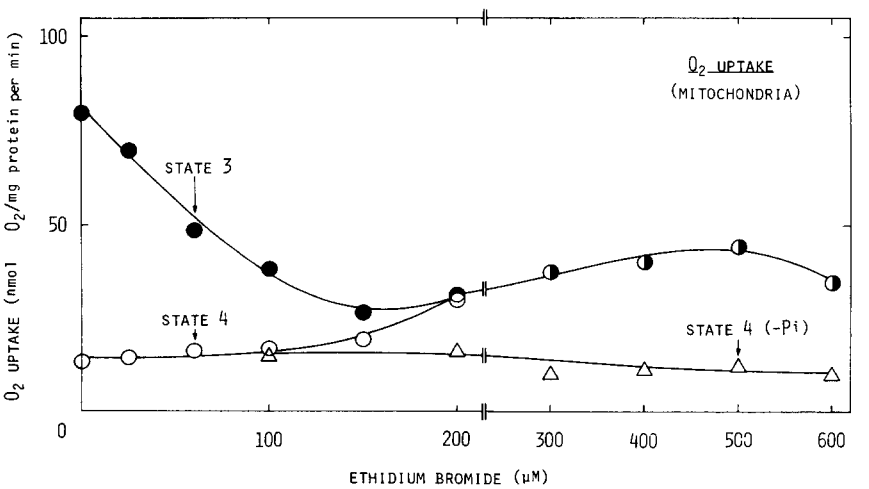

Fig. 2. Effect of ethidium bromide concentration on oxygen uptake by intact mitochondria. Conditions were as for Fig. 1 except that the indicated concentrations of ethidium bromide were used.

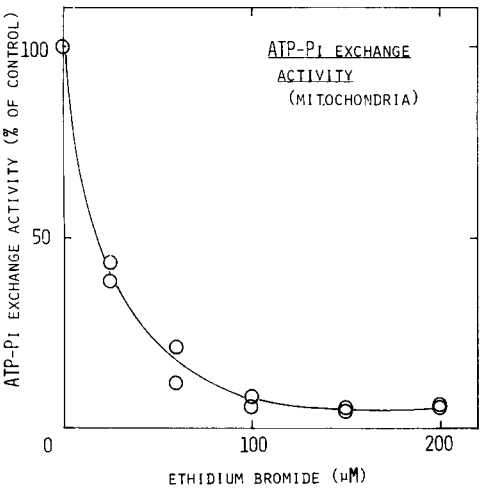

Fig. 3. Effect of ethidium bromide concentration on ATP-P_i exchange in intact mitochondria. Intact mitochondria (1 mg protein/ml) were preincubated for 5 min at 25°C in the presence of 4 mM ATP, 1 mM EDTA, 12 mM $MgCl_2$, 75 mM KCl, 50 mM sucrose, 50 mM Tris and various amounts of ethidium bromide, in a final volume of 1.5 ml at pH 7.4. The reaction was started by adding 15 μmol of potassium phosphate, containing 1.4×10^5 cpm of $^{32}P_i$, and was stopped 10 min later by adding 0.5 ml of 40% trichloroacetic acid.

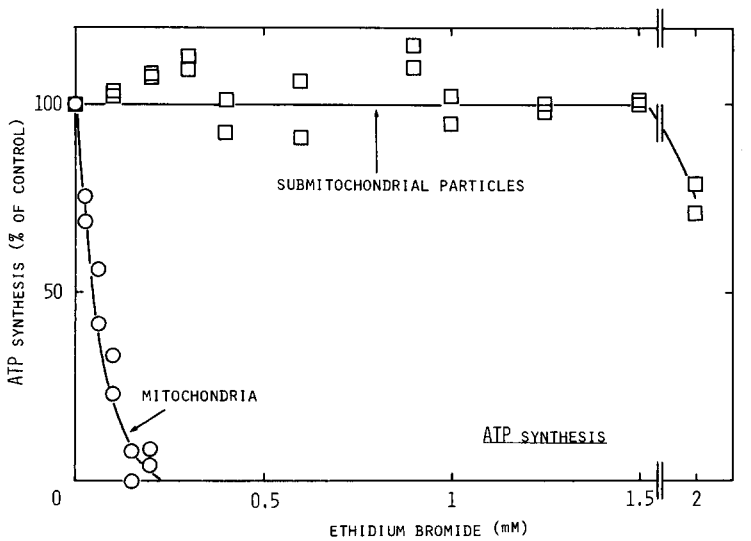

Fig. 4. Effect of ethidium bromide concentration on ATP synthesis in intact mitochondria and submitochondrial particles. Intact mitochondria (1 mg protein/ml) were preincubated for 5 min at 25°C in the presence of 10 mM succinate, 2 μg of rotenone, 0.5 mM ADP, 10 mM glucose, 0.1 mg of hexokinase, 5 mM $MgCl_2$, 2 mM EDTA, 15 mM KCl, 50 mM sucrose, 25 mM Tris and various amounts of ethidium bromide, in a final volume of 1.5 ml at pH 7.4. Submitochondrial particles (1 mg protein/ml) were preincubated for 5 min at 25°C in the presence of 10 mM succinate, 2 μg of rotenone, 0.5 mM ADP, 10 mM glucose, 0.1 mg of hexokinase, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM GSH, 10 mM Tris and various amounts of ethidium bromide, in a final volume of 1.5 ml at pH 7.4. The reaction was started by adding 30 μmol of potassium phosphate, containing about $5 \cdot 10^5$ cpm of $^{32}P_i$, and was stopped 6 min later by adding 0.5 ml of 40% trichloroacetic acid. The ATP/O ratios (esterified phosphate/oxygen uptake) of intact mitochondria and submitochondrial particles were 1.9 and 0.8, respectively.

ATP synthesis in submitochondrial particles, which are inside-out relative to the membranes of intact mitochondria [9—21], although 2 mM ethidium bromide partially inhibited ATP synthesis in the particles. Table I shows that ethidium bromide incorporated inside the submitochondrial particles inhibited ATP synthesis in the particles. Table I also shows that the dye incorporated inside the particles was not released in the suspending medium even by the washing of the particles (144 000 × *g* for 30 min). The relationship between the amount of ethidium bromide incorporated inside the submitochondrial particles and the decreasing rate of the activity of ATP synthesis in the particles corresponds approximately with the relationship between those in mitochondria (Figs. 4 and 7). Therefore, the inhibitory effect of the dye on ATP synthesis in intact mitochondria is not due to modification of the transport systems for respiratory substrates, inorganic phosphate or adenine nucleotide [35]. This explanation is also supported by the facts that energy-dependent binding of ethidium bromide to the mitochondria occurs with either respiratory substrates or ATP [5].

Effect of ethidium bromide on energy-dependent H^+ ejection from intact mitochondria

Intact mitochondria catalyze energy-dependent H^+ ejection [36—38]. Figs. 5 and 6 show that ethidium bromide also inhibited the initial rates of H^+ ejection from intact mitochondria energized with either succinate or ATP. These inhibitions are clearly different from those of well-known inhibitors of energy transduction in oxidative phosphorylation, such as oligomycin [36,37,39,40], dicyclohexylcarbodiimide [39,40] and aurovertin [40], because the latter

TABLE I

EFFECT OF INCORPORATION OF ETHIDIUM BROMIDE INSIDE THE SUBMITOCHONDRIAL PARTICLES ON ATP SYNTHESIS IN THE PARTICLES

Ethidium bromide was introduced into the submitochondrial particles as follow. The mitochondria (1 mg protein per ml) were incubated for 5 min at about 25°C in medium (pH 7.4) containing 200 μM ethidium bromide (Expt. 1) or 300 μM the dye (Expt. 2)/10 mM succinate/0.36 μg of rotenone per mg protein/5 mM $MgCl_2$/2 mM EDTA/15 mM KCl/50 mM sucrose/25 mM Tris. The resulting suspension was rapidly cooled to about 0°C and then centrifuged at 12 000 × *g* for 10 min. The precipitate was used as the starting material for preparing submitochondrial particles as described in the Materials and Methods. Control particles were obtained similarly, but without ethidium bromide treatment. Protein was determined by the biuret method, measuring absorbance at 650 nm. Amount of ethidium bromide incorporated inside the particles was estimated from its absorbance spectrum using a Hitachi Model 556 two-wavelength, double-beam spectrophotometer. Other experimental conditions were as for Fig. 4.

	Submitochondrial particles	ATP synthesis (nmol ATP/mg protein per min)	%	Incorporated Ethidium bromide (nmol/mg protein)
Expt. 1	Control particles	47.2	100	—
	Particles containing ethidium bromide	23.3	49.4	66.2
Expt. 2	Control particles	56.1	100	—
	Particles containing ethidium bromide *	7.3	13.0	186

* The mitochondria (5 mg protein/ml) were sonicated in the presence of 300 nmol ethidium bromide per mg protein.

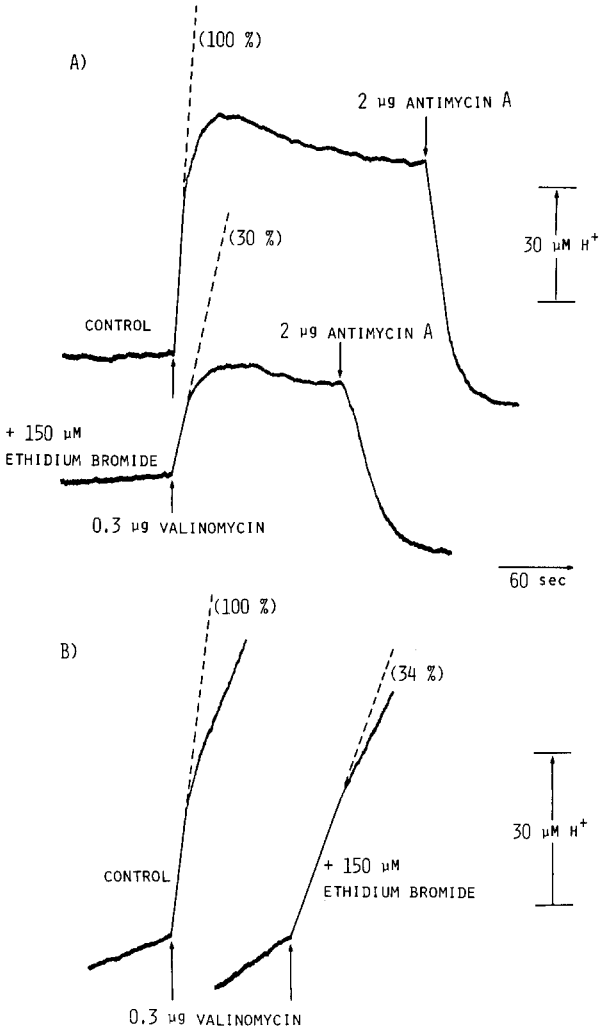

Fig. 5. Effect of ethidium bromide on energy-dependent H^+ ejection from intact mitochondria. In A, intact mitochondria (1 mg protein/ml) were preincubated for 5 min in the presence of 10 mM succinate, 2 µg of rotenone, 120 mM LiCl, 10 mM KCl, 10 mM Tris and 150 µM ethidium bromide, in a final volume of 3 ml at pH 7.15. In B, intact mitochondria were preincubated for 5 min in the presence of the same components as for A except that 2 mM ATP and 2 µg of antimycin A were used in place of succinate and rotenone. Valinomycin and antimycin A were added as indicated. The reaction was followed with a Hitachi-Horiba Model F-7 Expanded Scale pH meter at 18°C.

inhibit H^+ ejection from the mitochondria which is dependent on ATP, but not that dependent on electron transfer.

We found that the binding of ethidium to intact mitochondria energized by succinate caused ejection of protons into the suspending medium and this ejection of protons increased to the level of valinomycin (+ KCl)-dependent H^+ ejection with increase in the concentration of dye added (unpublished observation). We also found that the total amounts of protons ejected on addition of ethidium bromide and on subsequent addition of valinomycin (+ KCl)

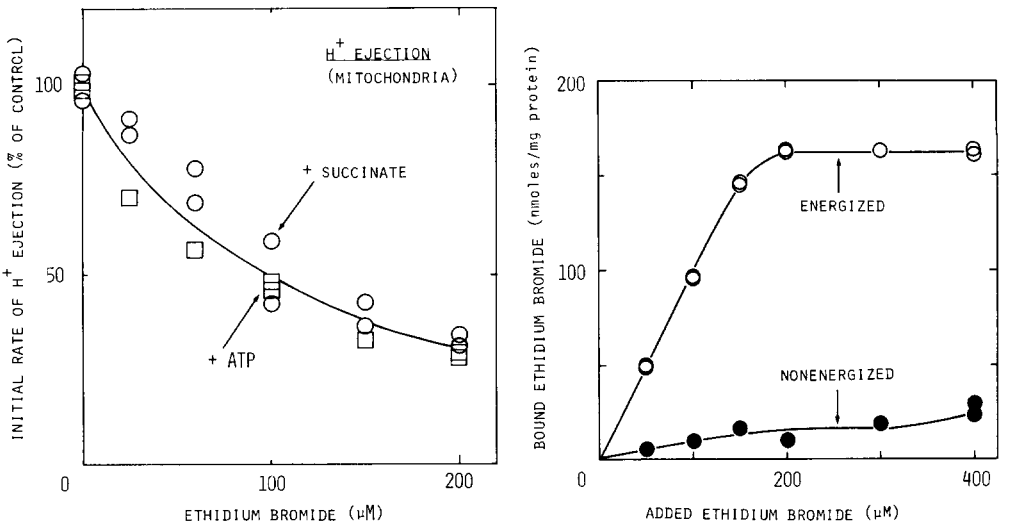

Fig. 6. Effect of ethidium bromide concentration on energy-dependent H^+ ejection from intact mitochondria. Conditions were as for Fig. 5 except that the indicated concentrations of ethidium bromide were used.

Fig. 7. Binding of ethidium bromide to mitochondria. The conditions were as described in Materials and Methods. The non-energized state of mitochondria was induced by further addition of 1.5 μM CCCP.

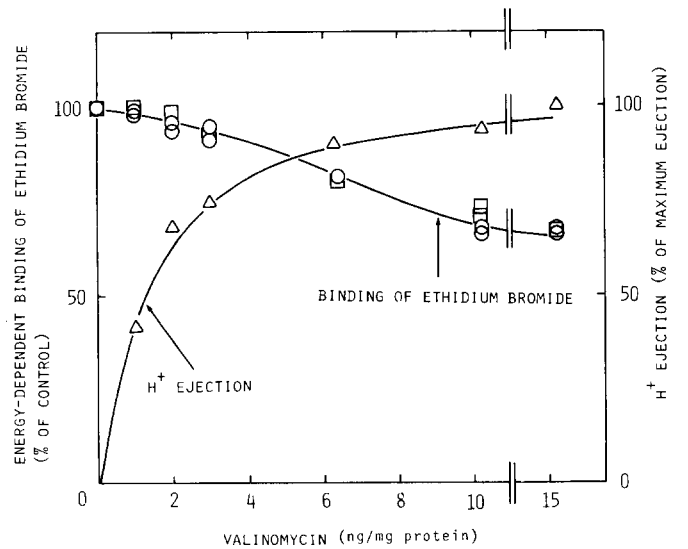

Fig. 8. Effects of valinomycin (+ KCl) on energy-dependent binding of ethidium bromide to mitochondria and energy-dependent H^+ ejection from the membrane. The conditions of binding of ethidium bromide were as for Fig. 7 except that valinomycin at the indicated concentration was also added before (○) or after (□) the energization of mitochondrial membrane in the presence of 20 mM KCl, 100 μM ethidium bromide and 10 mM succinate at pH 7.1. The pH change (△) was followed with a Hitachi-Horiba Model F-7 Expanded Scale pH meter. The conditions were the same as for ○ except that ethidium bromide was omitted. The amount of H^+ ejected from energized mitochodria in the presence of 15 ng of valinomycin per mg protein was 94.0 nmol per mg protein.

to energized mitochondria were constant, irrespective of the amount of the dye added (unpublished observation).

Energy-dependent binding of ethidium bromide to intact mitochondria

Fig. 7 shows that the saturation level of ethidium bromide bound to the energized mitochondria coincided approximately with the amount of the dye required to inhibit ATP synthesis completely (Fig. 4). When 50 μM ethidium bromide, which caused 50% inhibition of ATP synthesis in mitochondria (Fig. 4), was added in the energized mitochondrial suspension, 1.04 μM of dye was found in the suspending medium (Fig. 7). This amount of dye was determined using the fluorescence method described in the Materials and Methods. Fig. 8 shows that the effect of valinomycin (+ KCl) on energy-dependent binding of ethidium was not correlated with the effect of valinomycin (+ KCl) on energy-dependent H^+ ejection, and that valinomycin (+ KCl) only partially inhibited the energy-dependent binding of ethidium. These results differ from those on the binding to mitochondria of biguanides [41—43], which are inhibitors of energy transduction.

These results clearly show that the energy-dependent binding of ethidium is not due to electrophoretic transport of the dye through the mitochondrial inner membrane down the membrane potential [14,22—24]. These results could be due to the specific binding of ethidium to hydrophobic components on the surface of the C-side of the membranes.

Discussion

The present experiments showed that ethidium bromide inhibited energy transduction of oxidative phosphorylation in intact mitochondria but not in submitochondrial particles, which are inside-out relative to the membranes of intact mitochondria [9—21].

The sidedness of the inhibition could be explained by supposing that ethidium penetrates the membranes, becoming concentrated inside the mitochondria (minus inside), and it is extruded from the particles (plus inside) electrophoretically, i.e., in a membrane potential ($\Delta\psi$)-dependent fashion. A $\Delta\psi$ value of approx. 140 mV, which has been calculated to be present in state 4 mitochondria [44], would be consistent with an ethidium gradient of 235 : 1. For example, when 50 μM ethidium bromide, which caused 50% inhibition of ATP synthesis in mitochondria (Fig. 4), was added to the energized mitochondrial suspension, 1.04 μM of the dye was found in the suspension medium (Fig. 7). Thus there must have been 245 μM of dye in the mitochondrial matrix. Accumulation of this dye in the mitochondrial matrix might inhibit ATP synthesis by acting on oxidative and phosphorylative enzymes on the side of the membrane facing the matrix. If this explanation is valid, addition of 245 μM ethidium bromide to the submitochondrial suspension should cause 50% inhibition of ATP synthesis in the particles.

However, this explanation does not account satisfactorily for various observations, including the following. (1) Ethidium bromide at concentrations of up to 1.5 mM did not inhibit ATP synthesis in submitochondrial particles (Fig. 4). (2) Ethidium bromide incorporated inside the submitochondrial

particles inhibited ATP synthesis in the particles (Tabel I). (3) The saturation level of ethidium bound to energized mitochondria correspond with the amount of dye required to inhibit ATP synthesis completely (Figs. 4 and 7). (4) Decrease of the membrane potential by valinomycin (+ KCl) only slightly inhibited the energy-dependent binding of ethidium bromide to the mitochondria (Fig. 8).

The present results clearly show that ethidium bromide inhibited energy transduction in oxidative phosphorylation by acting on the C-side of the inner mitochondrial membrane, and that it had no inhibitory activity on the M-side of the membrane. The results also suggest that ethidium bromide did not penetrate the inner mitochondrial membranes rapidly. We propose that this new type of inhibitor should be called an "anisotropic inhibitor of energy transduction".

Our conclusion is consistent with the following anisotropic charge model. The anisotropic distribution of electric charge in the inner mitochondrial membrane [1,2] (in the energized membrane, the C-side of the membrane is more negative than the suspending medium, while the M-side is more positive than the matrix) is an intermediary high energy state on the main pathway of oxidative phosphorylation, and the energy-dependent binding of ethidium cation to the membrane inhibits ATP synthesis by neutralizing the negative charges created on the surface of the C-side of the membrane.

This conclusion is supported by the present results described above and the following more recent findings (unpublished observations). (1) Like ethidium bromide, acriflavine [45] and tetraphenylarsonium, which are positively-charged amphipathic molecules, inhibited energy transduction of oxidative phosphorylation in intact mitochondria, but not in submitochondrial particles. However, Montal et al. [46] reported that tetraphenylarsonium uncouples rat liver mitochondria but not submitochondrial particles. The discrepancy between these results and ours will be discussed in detail in a later paper. (2) Anilinonaphthalene sulfonate, with a negative charge, inhibited remarkably ATP synthesis in submitochondrial particles, but inhibited only slightly that in intact mitochondria. (3) Anilinonaphthalene sulfonate and ethidium bromide have opposite effects on chloroplast grana membranes, which have the opposite polarity to that of intact mitochondrial membranes [47]. The former effects are similar to the effects of these compounds on oxidative phosphorylation in submitochondrial particles: anilinonaphthalene sulfonate inhibited energy transduction of photophosphorylation in chloroplast grana from spinach leaves, whereas ethidium bromide did not. (4) The binding of ethidium to intact mitochondria energized with succinate or ATP caused ejection of protons into the suspending medium. This finding could be explained as follows: ethidium cation binds electrostatically to a negative charge created on the C-side of the membrane and then the proton drawn electrostatically to the negative charge is released into the suspending medium to maintain the electroneutrality of the medium. This neutralization of the negative charges on the C-side by ethidium could be reason why ethidium acts as an energy linked probe for intact mitochondria [2—5] and also the dye inhibits energy transduction in intact mitochondria.

Note added in proof (Received May 2nd, 1978)

We found that the binding of positively charged ethidium to negatively charged tetraphenylboron caused a red shift in the absorbance maximum of ethidium, like the absorbance change of ethidium in the energized mitochondria [2]. We also found that tetraphenylboron reversed the inhibition of ATP synthesis by ethidium in mitochondria. However, positively charged tetraphenylarsonium did not exhibit such effects of tetraphenylboron described above. These findings clearly show that ethidium cations inhibit energy transduction of oxidative phosphorylation in mitochondria by binding to the negative charges created on the C-side of the membrane, as proposed in the present paper. This phenomenon will be discussed in detail in the following paper.

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