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EFFECT OF ETHACRYNIC ACID ON THE PERMEABILITY OF THE MITOCHONDRIAL MEMBRANE IN RAT LIVER MITOCHONDRIA

SALVATORE PASSARELLA and ERNESTO QUAGLIARIELLO

CNR Unit for the study of Mitochondria and Bioenergetics and Department of Biochemistry, University of Bari, 70126 Bari, Italy

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Abstract—The effects of the diuretic agent ethacrynic acid on some mitochondrial reactions have been investigated using rat liver mitochondria. Ethacrynic acid inhibits the oxidation of succinate in a competitive manner, whereas non-competitive inhibition by ethacrynic acid is found on the translocation of *cis*-aconitate and oxoglutarate as measured by fluorimetry. Ethacrynic acid causes mitochondrial swelling in solution of ammonium salts of several "non-penetrant" anions. Swelling depends on the reactivity of ethacrynic acid with thiols of the mitochondrial membrane. These results suggest a general ability of ethacrynic acid to inhibit mitochondrial carriers and influence the membrane conformation and permeability. These conclusions are discussed in the light of the effect of this diuretic agent on mitochondrial and cellular activity and of the location of thiols in the carriers of citric cycle intermediates.

Ethacrynic acid (2,3-dichloro-4[2′-methylenebutyryl]phenoxyacetic acid) is a potent diuretic drug and it is known to react with thiols. Several papers have been published on the effect shown by ethacrynate on kidney slices [1–4] and on enzymes [5–18]. Moreover it has been proposed that ethacrynate may bind a hypothetical energy-rich intermediate [19] and an energy-dependent permeation into mitochondria was also shown [20]; ethacrynate interferes with the mitochondrial respiratory control of Ehrlich ascites tumor cells [21], and it was suggested that it has an inhibitory action upon the electron transfer system and hence reduces the efficiency of ATP formation in rat kidney mitochondria [22]. However, knowledge about the molecular effect of this drug on the permeability properties of the mitochondrial membrane is very far from being exhaustively clarified. We have shown the competitive nature of the inhibition by ethacrynate of the rate of malonate uptake in rat liver mitochondria [23] and inhibition of the adenine nucleotide carrier has been reported in rat kidney mitochondria [24]. In view of the importance of permeability processes in mitochondria bioenergetics and in cellular activity, we decided to investigate, the possible effect of this drug on the transport of citric cycle intermediates as well as the effect of ethacrynate on the general permeability properties of the membrane as revealed by photometric measurement of mitochondrial swelling.

This study allows us to distinguish two separate actions of ethacrynate on mitochondria: ethacrynate inhibits the translocation of some metabolic substrates and directly influences the permeability and the conformation of the mitochondrial membrane.

MATERIALS AND METHODS

Preparation of rat liver mitochondria. All studies utilized 200–250 g male Wistar rats. Mitochondria were isolated in 0.25 M sucrose, 20 mM Tris–HCl, pH 7.25 and 1 mM EGTA as previously described [25]. The final mitochondrial pellet was suspended in the same medium to give a protein concentration between 40 and 60 mg/ml.

Mitochondrial protein was determined by a modified biuret method [26].

Measurement of the rate of oxygen uptake. Mitochondrial oxygen uptake was measured polarographically at 26° with a Clark electrode using an incubation medium containing 0.25 M sucrose, 20 mM Tris–HCl pH 7.25, 1 mM EGTA and 2 μg/ml rotenone. The reaction was started by the addition of succinate in the presence or absence of sodium ethacrynate followed by the addition of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (final concentration 2 μM).

Fluorimetric assays. Changes in the ox/redox state of $NAD(P)^+$ were followed at 30° by fluorimetry using an Eppendorf photometer Model 1101 M equipped with the fluorescence apparatus.

Reduction: mitochondria were incubated in 1 ml of a medium containing 125 mM KCl, 20 mM Tris–HCl pH 7.0 and 2 mM inorganic phosphate. A typical experiment was carried out as follows: FCCP (final concentration 2 μM) was added to the mitochondria in order to oxidize the intramitochondrial nicotine nucleotides; when the oxidation was complete respiratory inhibitors such as rotenone (2 μg/ml) and antimycin (4 μg/ml) were added. Subsequently malate was added in order to activate tricarboxylate uptake (for ref. see [23]); little reduction was observed as result of this addition. Finally *cis*-aconitate was added, a rapid reduction of the intramitochondrial $NAD(P)^+$ occurred and the rate was calculated in arbitrary units. When present sodium ethacrynate was added to the mitochondria together with the substrate.

Oxidation: mitochondria were incubated at 30° in 1 ml of a medium containing 0.2 M sucrose, 20 mM *N*-2 - hydroxyethyl - piperazine - *N*′ - 2 - ethanesulfonate

(HEPES)–Tris pH 7.0, 10 mM NH_4Cl, 5 mM malonate, 2 mM arsenite and 2 μg rotenone. After 1 min incubation oxoglutarate was added; a rapid oxidation of the intramitochondrial NAD(P)H occurred and the rate was calculated in arbitrary units. When present sodium ethacrynate was added to the mitochondria together with the substrate.

Measurement of mitochondrial swelling. Mitochondrial swelling was monitored at 25° by recording the decrease in absorbance measured at 546 nm with an Eppendorf photometer Model 1101 M. Mitochondria were added to the appropriate solution and the absorbance was recorded as a function of time in the absence or presence of sodium ethacrynate. When necessary the pH of the solution was adjusted to neutrality by adding NH_4OH (in the case of solution of ammonium salts) or Tris.

In all the experiments, mitochondrial preparations with respiratory control less than three were discarded.

RESULTS

Effect of ethacrynate on oxygen uptake stimulated by succinate. Although inhibition by ethacrynate of the oxidation of succinate was reported previously [19], no attempt was made to explain the experimental findings in terms of a possible inhibition of the translocation of the substrate by ethacrynate; moreover the measurements of the oxygen uptake were made either by an oxipolarographic or a manometric method, after a 10 min incubation of mitochondria with the drug, by which time penetration of ethacrynate into matrix would have occurred [27]. Thus we investigated the inhibition of the oxidation of succinate by ethacrynate by adding substrate and inhibitor simultaneously to the mitochondrial suspension and immediately measuring the rate of oxygen uptake stimulated by the uncoupler FCCP. It should be noted that no significant change in the measurement was found if the uncoupler was added to the mitochondria just before addition on the substrate and the inhibitor. The nature of the inhibition was investigated by means of a double reciprocal plot (Fig. 1). By raising the concentration of succinate the degree of inhibition by 67 μM ethacrynate was decreased, but the V_{max} did not change in agreement, as would be expected for a competitive inhibition. The K_i value was 75 μM, in fair accordance with the K_i found for the inhibition of the rate of malonate uptake via the dicarboxylate carrier [23].

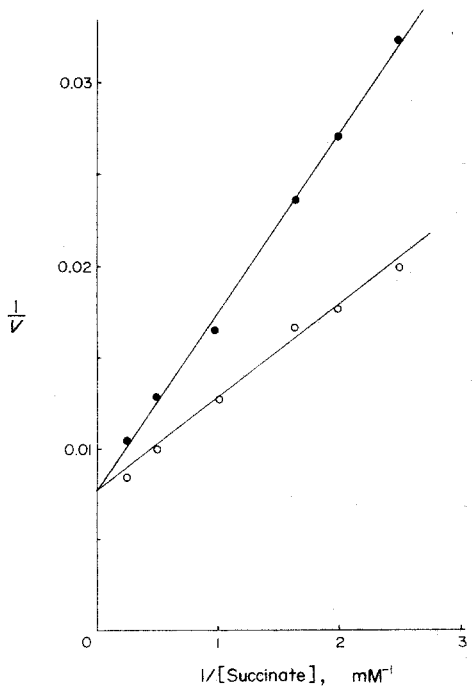

Fig. 1. Kinetic analysis of the inhibition of succinate oxidation by ethacrynate. The oxygen uptake was measured in the absence (○) or in the presence (●) of 67 μM ethacrynate. V is expressed as natoms O_2/min × mg protein. Mitochondrial protein was 2.7 mg.

Effect of ethacrynate on the activity of the tricarboxylate and oxoglutarate carriers. In rat liver mitochondria specific carriers are present which translocate citrate, *cis*-aconitate and isocitrate (the tricarboxylate carrier) or oxoglutarate and oxaloacetate [28, 29] (the oxoglutarate carrier). The inhibition by ethacrynate of the activity of these carriers was tested by using indirect methods, which have been reported to measure the permeation of substrate into mitochondria [30, 31]. The activity of the tricarboxylate carrier was investigated by following the change in the redox state of the intramitochondrial NAD(P)$^+$ induced by addition of *cis*-aconitate as described in Materials and Methods. Ethacrynate was found to inhibit the rate of *cis*-aconitate oxidation and the nature of the inhibition was investigated in the experiment reported in Fig. 2; the data are presented either as a double reciprocal plot (Fig. 2A) or as a Dixon plot (Fig. 2B).

The presence of 100 μM ethacrynate did not change the K_m value for the uptake of *cis*-aconitate, whereas the V_{max} decreased as could be expected for a non-competitive inhibition. The non-competitive nature of the inhibition was confirmed by studying the dependence of the rate of uptake of 3 mM or 20 μM *cis*-aconitate on increasing concentrations of ethacrynate. In both cases the K_i values were 75 μM.

In order to study the influence of ethacrynate on oxoglutarate transport, we measured the oxidation of intramitochondrial NAD(P)H induced by the addition of increasing concentrations of oxoglutarate (plus ammonium ions) in the absence or presence of 300 μM ethacrynate. The data are presented as a double-reciprocal plot (Fig. 3). The measurements were carried out in the presence of rotenone to block the NADH dehydrogenase, arsenite to inhibit the oxoglutarate dehydrogenase and malonate to activate oxoglutarate entry into the mitochondria [32]. Ethacrynate was found to inhibit the NADH oxidation; the K_m value for oxoglutarate uptake did not change, whereas the V_{max} decreased as expected for non-competitive inhibition. The K_i value was 340 μM.

Swelling of rat liver mitochondria induced by ethacrynate. It was previously reported that in mitochondria of different tissue ethacrynate interferes with phosphorylation reactions [19–22, 33]. Given that it has been recognised that interference with phosphorylation often, but not always, leads to extensive structural modification of mitochondria, we have begun to investigate the possible effects of ethacrynate on the conformation of the mitochondrial membrane by testing

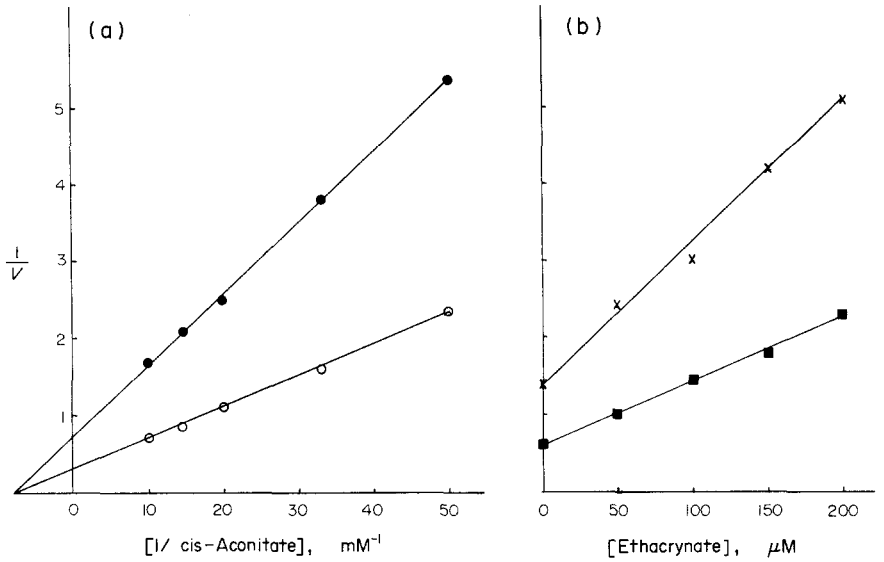

Fig. 2. Kinetic analysis of the inhibition of *cis*-aconitate uptake by ethacrynate. A, Double reciprocal plot of *cis*-aconitate uptake in the absence or in the presence of ethacrynate. The rate of $NAD(P)^+$ reduction was measured in the absence (○) or in the presence (●) of 100 μM ethacrynate. Mitochondrial protein was 1.7 mg. B, Dixon plot of the inhibition of *cis*-aconitate uptake by ethacrynate. The rate of $NAD(P)^+$ reduction induced by 3 mM (■) or 20 μM (×) *cis*-aconitate is measured in the absence or in the presence of increasing concentrations of ethacrynate. Mitochondrial protein was 2.8 mg.

changes in volume of the mitochondria suspended in several isotonic media.

The optical density of mitochondria suspended in sucrose solution does not change in the presence of 0.6 mM ethacrynate, even after 30 min of incubation.

Chappell and Haarhoff [34] have reported that no swelling occurs when mitochondria are suspended in 100 mM ammonium chloride, and this finding was taken to show the impermeability of mitochondria to chloride ions. On the other hand we have found that if 0.6 mM ethacrynate is added to mitochondria suspended in 100 mM NH_4Cl, a rapid and extensive change of optical density takes place (Fig. 4). In view of the chemical analogies of chloride, bromide and iodide ions, the effect of ethacrynate on the absorbance changes of mitochondria suspended in 100 mM ammonium salts of these anions was compared (Fig. 5). No swelling was found in the absence of the diuretic agent, but the presence of 0.6 mM ethacrynate rapidly decreased the absorbance of the mitochondrial suspension. The maximum rate of swelling was found with ammonium bromide solutions. In order to gain further insight into the effect of ethacrynate on the absorbance of mitochondria suspended in chloride solutions, the ammonium ion was replaced with the small ion Li^+ or with the large Cs^+. Thus swelling of mitochondria suspended in 100 mM lithium and cesium chlorides was tested in the absence or in the presence of 0.33 and

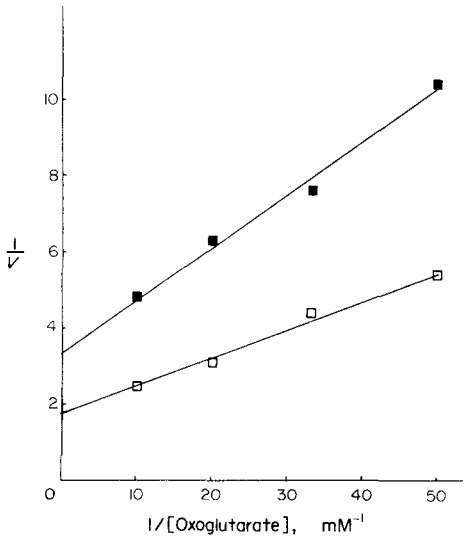

Fig. 3. Kinetic analysis of the inhibition of oxoglutarate uptake by ethacrynate. The rate of NAD(P)H oxidation was measured in the absence (□) or in the presence (■) of 300 μM ethacrynate. Mitochondrial protein was 2.2 mg.

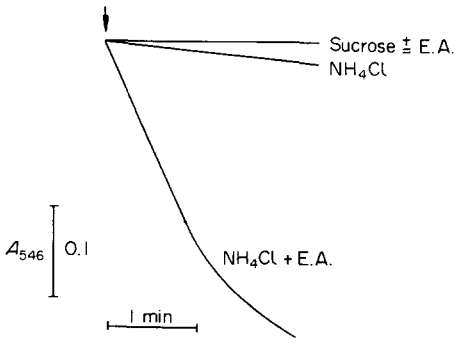

Fig. 4. Swelling of isolated liver mitochondria induced by ethacrynate. Mitochondria (2.4 mg protein) were suspended in 0.25 M sucrose or 100 mM NH_4Cl pH 7.0 as indicated. At the point marked by an arrow the suspension was made 0.63 mM with respect to ethacrynate (E.A.). Temperature: 22°.

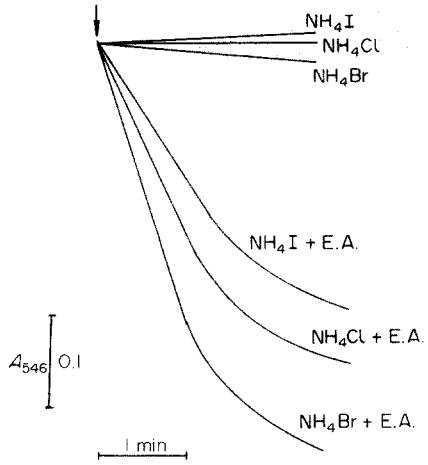

Fig. 5. Swelling of isolated liver mitochondria in ammonium halide solutions. Mitochondria (2 mg protein) were suspended in 100 mM NH_4Cl, NH_4Br or NH_4I, pH 7.0. Where indicated by an arrow the suspension was made 0.63 mM with respect to ethacrynate (E.A.).

0.82 mM ethacrynate (Fig. 6). Swelling was found for both salts, increasing with the concentration of the diuretic agent. Mitochondrial swelling induced by ethacrynate also occurs in calcium, potassium and sodium chlorides, whereas in contrast no change in absorbance of mitochondria suspended in magnesium chloride was found after the addition of ethacrynate.

Effect of cysteine on the mitochondrial swelling induced by ethacrynate. In order to investigate the mechanism of swelling induced by ethacrynate, the effect of the addition of cysteine to the suspension was tested in several experiments. In the typical experiment shown in Fig. 7, cysteine was added to mitochondria suspended in ammonium chloride and no change in absorbance was found. If, on the other hand, cysteine was added to swelling mitochondria after ethacrynate addition, strong inhibition of swelling was found (Fig. 7B); cysteine was also found to prevent the swelling if added together with ethacrynate (Fig. 7A); at a cysteine/ethacrynate ratio equal to 5, no significant swelling was found in ammonium chloride. The same results were found with all tested salts.

It should be noted that no swelling was shown by mitochondria suspended in ammonium chloride after addition of mersalyl or *N*-ethylmaleimide under the same experimental conditions.

DISCUSSION

In this paper evidence is given concerning the ability of ethacrynate to inhibit the carriers which mediate the translocation of citric cycle intermediates in rat liver mitochondria.

Ethacrynate permeates into rat liver mitochondria, as demonstrated by measuring the inactivation of reduced intramitochondrial glutatione [27], and its penetration is greatly stimulated by the presence of a respiratory substrate. The activation of permeation is completely inhibited by an uncoupler or inhibitor of the respiratory chain [20]. Under the experimental conditions used in the present work, the inhibition of succinate uptake could be attributed to the inhibition of transport since measurements were carried out in the first seconds after the addition of ethacrynate when only the external thiols of the membrane were likely to be bound. Moreover the oxidation was stimulated by the uncoupler, decreasing in this way the penetration of ethacrynate in the mitochondrial matrix. Finally it should also be noted that the rate of oxidation of succinate plus rotenone is controlled by the uptake of succinate [35]. The competitive inhibition found for succinate oxidation confirms the previously reported results (see [23]), and suggests the presence of -SH groups at or near the specific dicarboxylate binding site in the dicarboxylate carrier [36]. The non-competitive inhibition by ethacrynate of the tricarboxylate and oxoglutarate carrier suggests that thiols in these carriers are located far from the substrate binding site [23, 37].

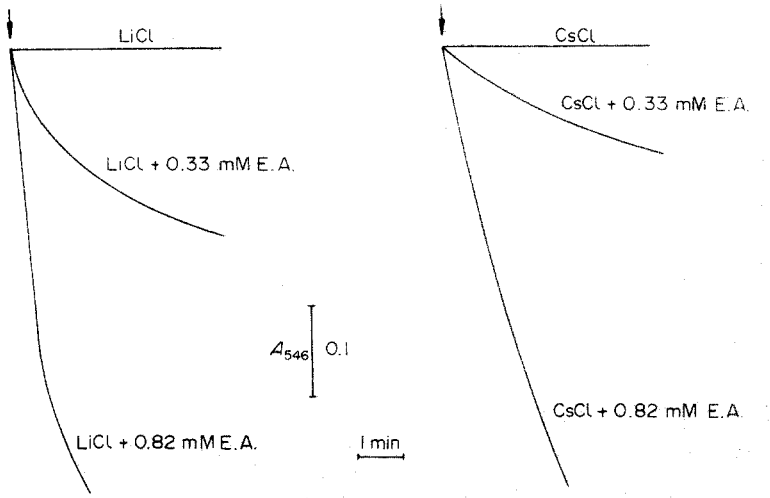

Fig. 6. Swelling of isolated liver mitochondria in lithium or cesium chloride solution, induced by ethacrynate. Mitochondria (3.3 mg protein) were suspended in 100 mM LiCl or CsCl, pH 7.0. Where indicated by the arrow the suspension was made 0.33 or 0.82 mM with respect to ethacrynate (E.A.).

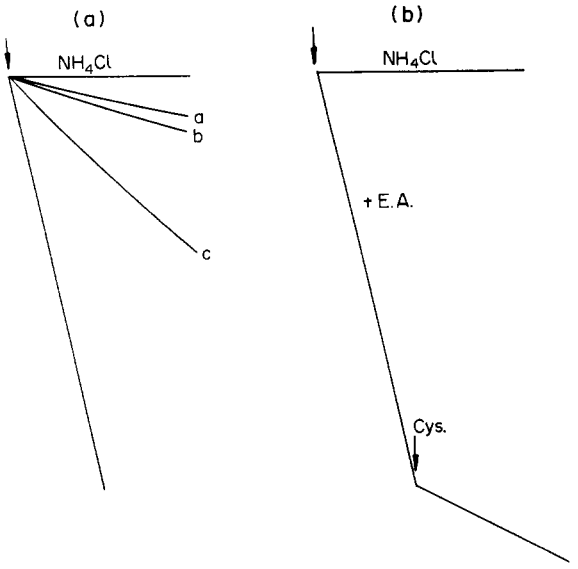

Fig. 7. Effect of cysteine on the swelling of isolated mitochondria in ammonium chloride solution induced by ethacrynate. A, Prevention by cysteine of swelling induced by ethacrynate. Mitochondria (2.5 mg protein) were suspended in 100 mM NH_4Cl, pH 7.0. In a, b and c, cysteine was added at the following concentrations: a, 1 mM; b, 0.33 mM; c, 0.17 mM. After 1 min at the point marked by an arrow, the suspension was made 0.33 mM with respect to ethacrynate. B, Inhibition by cysteine of swelling induced by ethacrynate. Mitochondria (2.5 mg protein) were incubated in 100 mM NH_4Cl, pH 7.0. Where indicated at the points marked by arrows the suspension was made 0.63 mM and 8.3 mM with respect to ethacrynate (E.A.) and cysteine (Cys.).

Benzene-1,2,3-tricarboxylate, a specific inhibitor of the tricarboxylate carrier (for ref. see [23]), protects the carrier against the inhibition by ethacrynate, as has already been reported for inhibition by mersalyl [23]. This finding confirms that ethacrynate inhibits *cis*-aconitate transport; in fact no prevention by the impermeable anion benzene-1,2,3-tricarboxylate could be shown on the inhibition of the activity of the intramitochondrial enzymes by ethacrynate. The inhibition of NAD(P)H oxidation is due to the inhibition of the uptake of oxoglutarate; in fact, if oxoglutarate was accumulated in the mitochondrial matrix before the addition of ethacrynate, or if oxoglutarate-loaded mitochondria were used, no inhibition was found when the reaction was started by the addition of ammonium ions. These pieces of evidence demonstrate that the uptake is the rate limiting step in the measured process and that ethacrynate inhibits the substrate penetration.

It should be noted that ethacrynate inhibits swelling of rat liver mitochondria in ammonium phosphate solutions [33], as well as the adenine translocase function in rat kidney mitochondria [24]; thus the ability of ethacrynate to inhibit substrate translocation across the mitochondrial membrane should be considered a major characteristic of the mechanism of action of this drug. It is worth considering that impairment of oxidative metabolism due to this inhibitor would have a profoundly deleterious effect on the cellular functions, and the activity of the mitochondrial carriers appears to be quite essential in several metabolic processes involving cytosol–mitochondria interaction such as fatty acid synthesis or transfer of reducing equivalents from cytosol to mitochondria and vice versa.

Several observations suggest that both -SH and S-S-groups are critical in the membrane properties [38–42]. This point of view is supported by the finding that Ag^+ ions and other heavy metal ions capable of forming mercaptides with protein also cause mitochondrial swelling [43]. The mitochondrial swelling induced by ethacrynate supports these conclusions. The changes of mitochondrial conformation revealed by swelling are dependent on the ability of ethacrynate to react with -SH groups. In fact cysteine does not allow swelling to occur and strongly inhibits this process when it is in progress. It should be noted, however, that ethacrynate does not cause irreversible swelling; the addition of ATP, in fact, produces rapid shrinkage of the swollen mitochondria. Mitochondrial swelling induced by ethacrynate appears to be strictly dependent on the composition of the medium. No swelling was found in non-ionic media, such as sucrose or, for instance, in magnesium chloride. The possible swelling induced by ethacrynate was tested in ammonium salt solutions of non-penetrant anions such as fumarate, methylfumarate and maleate [34], but no swelling was found. These findings might suggest that swelling is dependent on the increase of the permeability of the mitochondria to certain ions. It should be noted that ion movements induced by ethacrynate have been reported previously [33]; thus the results reported here may be interpreted in favour of the ability of ethacrynate to induce permeation of chloride, bromide and iodide and some impermeable ions such as Cs^+.

In conclusion, it is clear that the mechanism of action of the diuretic ethacrynate should be further investigated *in vivo* and *in vitro*, taking into consideration the influence of this compound on reactions involving the permeability properties of mitochondria either with respect to carrier-mediated translocations (at lower concentrations) or on the general membrane properties.

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