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INFLUENCE OF THE ENERGETIC STATE OF RAT LIVER MITOCHONDRIA ON THE SENSITIVITY OF THE PHOSPHATE CARRIER TOWARDS SH REAGENTS

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SUMMARY

Phosphate transport into rat liver mitochondria was measured by the swelling technique in 0.1 M ammonium phosphate. Energized or non-energized mitochondria were preincubated with different thiol reagents and evidence is given that with a slow-reacting thiol reagent, ethacrynate, the inactivation of the phosphate carrier is obtained when mitochondria are energized, while poor or no inactivation occurs when mitochondria are non-energized or preincubated with P_i. Moreover, the inactivation depends on the presence of Mg^{2+} and on the nature of the substrate.

Some comparative essays were done using *N*-ethylmaleimide as a thiol reagent, but no energy-linked variation of *N*-ethylmaleimide inhibition on phosphate transport was obtained.

Taking into account the fact that both thiol-reagents incorporation into rat liver mitochondria is stimulated by the presence of substrate, the different behaviour of these two thiol-reagents towards P_i transport is discussed on the basis of their different reactivity with SH groups.

INTRODUCTION

It is generally accepted that P_i penetration through the inner mitochondrial membrane is supported by two different carrier systems: one is a P_i/OH^- antiport system which depends on a transmembrane pH gradient [1–4] and which is inhibited by thiol reagents [1, 5–7], the other being an exchange system between P_i and some dicarboxylates [2, 8], insensitive to NEM [6] but inhibited by mercurials and some dicarboxylate analogs such as *n*-butylmalonate.

However, it was shown recently that both systems (the so-called P_i carrier and

Abbreviations: Ethacrynic acid, 2,3-dichloro-4(2′-methylene butyryl)-phenoxyacetic acid; NEM, *N*-ethylmaleimide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EGTA, ethylene glycol bis(β-amino-ethyl-ether)-*N*,*N*′-tetraacetic acid.

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dicarboxylate carrier) could share a similar component with an essential -SH group while their specific carrier activity would be due to the presence of specific sub-units for each system [4].

Moreover, several authors [9, 11] have shown that the sensitivity of the P_i carrier towards -SH reagents depends on the composition of the incubation mixture: presence of substrate, P_i, ionophore antibiotics, uncoupling agents, etc. By using ethacrynate, a slow thiol alkylating reagent which penetrates into mitochondria [12], and studying its effects on rat liver mitochondria [13–15], we have shown that some of these effects and ethacrynate incorporation into rat liver mitochondria depend on their metabolic state.

This led us to the present study; the influence of ethacrynate on P_i transport (estimated by the swelling technique), with mitochondria in different energetic states. A comparative study was carried out with NEM.

The reported results show unequivocally that the P_i carrier sensitivity towards ethacrynate is strongly dependent on the energetic state of the mitochondria: when mitochondria are energized (presence of substrate) ethacrynate strongly inhibits P_i penetration, while when mitochondria are deenergized (absence of substrate or presence of uncoupling agents) poor or no inhibition is obtained.

These results are in good agreement with previous findings [14], where we have shown that ethacrynate incorporation into rat liver mitochondria is stimulated by substrate-induced energization.

However, it appears that with NAD-linked substrates substrate concentration is determinant: with low substrate concentrations one gets an increase of both inhibition of swelling by ethacrynate and ethacrynate incorporation into rat liver mitochondria, while with higher substrate concentrations these substrate-induced effects decrease.

With succinate, its stimulating effects on inhibition of swelling by ethacrynate and on ethacrynate incorporation into rat liver mitochondria is independent of its concentration.

Moreover, our results show that for ethacrynate concentrations between 30 and 50 nmol · mg^{-1} of proteins, there is a correlation between substrate-stimulated ethacrynate binding and substrate-stimulated inhibition of swelling of rat liver mitochondria in ammonium phosphate, which is not the case for ethacrynate concentrations higher than 50 nmol · mg^{-1} of proteins.

MATERIALS AND METHODS

Isolation of rat liver mitochondria

Rat liver mitochondria were prepared according to Weinbach [16] and mitochondrial proteins estimated by the quick Biuret method after solubilization in 0.2 % sodium cholate [17].

Osmotic swelling measurement

Rat liver mitochondria (approx. 5 mg protein) were preincubated for 11 min at 30 °C in the standard medium: 62 mM sucrose/8 mM $MgCl_2$/50 mM KCl/20 mM glycylglycine pH 7, final volume 1 ml. When present, antimycin (10 μg), rotenone (8 μg), 1.25 μM CCCP, 5 mM *n*-butylmalonate, or 1 mM P_i were added to the medium before rat liver mitochondria; substrates and thiol reagents (ethacrynate or NEM) were added 30 s and 1 min respectively, after rat liver mitochondria.

At the end of preincubation an aliquot (100 μl) of the mixture was quickly mixed to 1.9 ml of 0.1 M ammonium phosphate +0.5 mM EGTA. Absorbance variations were followed at 546 nm with a recording photometer. The mitochondrial swelling rate was determined by measuring the absorbance variation obtained after the first 12 s.

Ethacrynate and NEM incorporation measurement

Rat liver mitochondria (4.5–6 mg protein) were incubated 10 min at 30 °C, pH 7 in 1 ml of the standard medium, with 10 mM substrate when present, and [^{14}C]ethacrynate (specific activity 100–200 dpm · nmol^{-1}) or [^{3}H]NEM (specific activity 200 dpm · nmol^{-1}). 0.5 ml samples were treated as previously described [14]. [^{14}C]ethacrynic acid and [^{3}H]-NEM were prepared by the Commissariat à l'Energie Atomique, Saclay, France.

Polyacrylamide gel electrophoresis

Mitochondrial proteins were analysed by SDS polyacrylamide gel electrophoresis as described by Coty and Pedersen [18]; proteins were stained with Coomassie Blue. Gels were analysed for radioactivity by slicing (2 mm) and digesting in 0.2 ml 30 % H_2O_2 overnight at 70 °C, followed by counting in a Triton X-100/toluene/PPO/POPOP mixture.

RESULTS

Different sensitivity of the phosphate carrier to ethacrynate and NEM

Fig. 1 shows that the phosphate carrier is much more sensitive to NEM than to ethacrynate; with NEM the inhibition increases with concentration and 90 % inhibition is obtained with 20 nmol · mg^{-1} of proteins. With ethacrynate, after an increase of inhibition which is maximum (nearly 40 %) for 50 nmol · mg^{-1} of proteins, inhibition decreases for higher concentations.

Influence of substrate on swelling inhibition by ethacrynate and ethacrynate incorporation: comparison with NEM

Fig. 2A shows that the presence of substrate (succinate 10 mM) significantly increases ($\times 2$) swelling inhibition by ethacrynate; maximum inhibition is reached for 50 nmol · mg^{-1} of proteins, and higher concentrations give lower inhibition.

Fig. 2B shows that the presence of substrate increases [^{14}C]ethacrynate incorporation somewhat for ethacrynate amounts > 30 nmol · mg^{-1} of proteins. This stimulation of ethacrynate incorporation is not obtained by adding ATP instead of substrate (not shown).

By using SDS polyacrylamide gel electrophoresis to separate mitochondrial proteins, it appears that when substrate is present during preincubation with [^{14}C] ethacrynate, the labelling of some mitochondrial proteins is significantly increased (Fig. 3).

In Table I it appears that while energization of mitochondria induced by substrate oxidation increased NEM incorporation, inhibition of swelling was not significantly enhanced.

With dihydroethacrynate (unable to react with -SH groups) there was no inhibition of swelling, whatever the conditions.

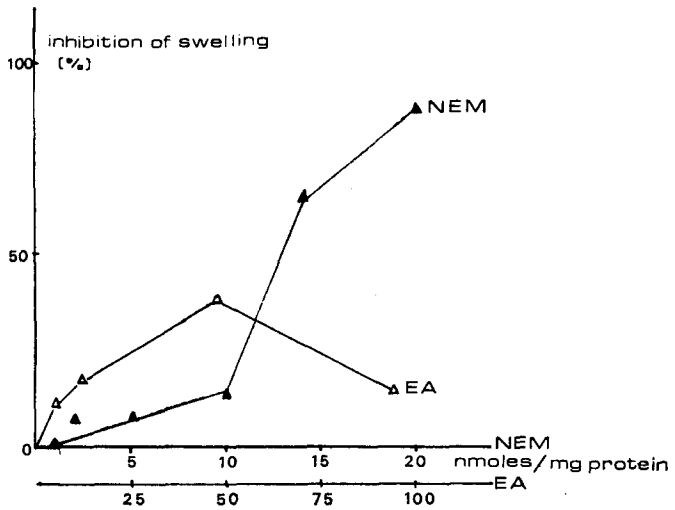

Fig. 1. Comparative effects of ethacrynic acid (EA) and NEM on osmotic swelling of mitochondria in ammonium phosphate. Mitochondria (5 mg) were preincubated without substrate for 10 min with ethacrynic acid or 2 min with NEM in 1 ml of the standard medium. An aliquot of 100 μl was used for measuring swelling at 546 nm.

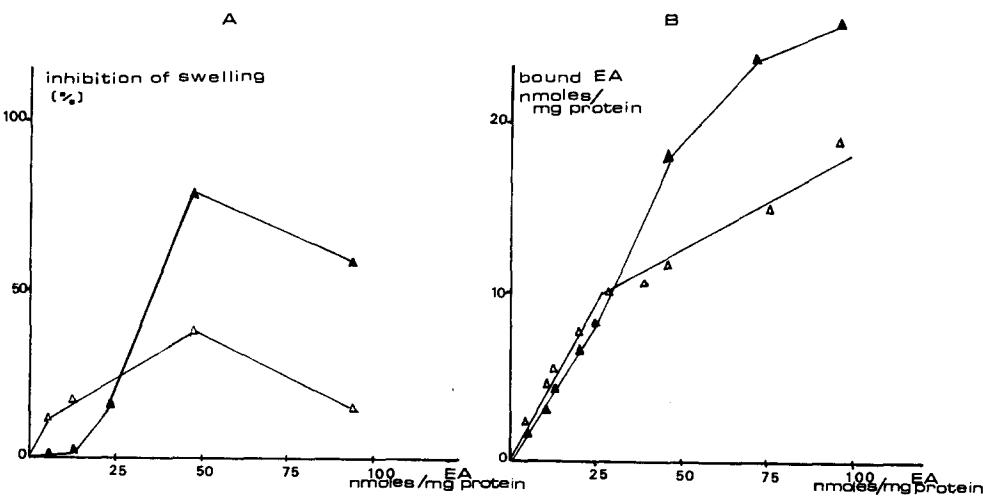

Fig. 2. Relation between inhibition of osmotic swelling (A) and [^{14}C]ethacrynate incorporation (B). Mitochondria (5 mg) were preincubated in 1 ml of the standard medium for 10 min at 30 °C, without substrate (△—△) or in the presence of 10 mM succinate (▲—▲). Osmotic swelling and bound ethacrynic acid (EA) were estimated as described under Materials and Methods.

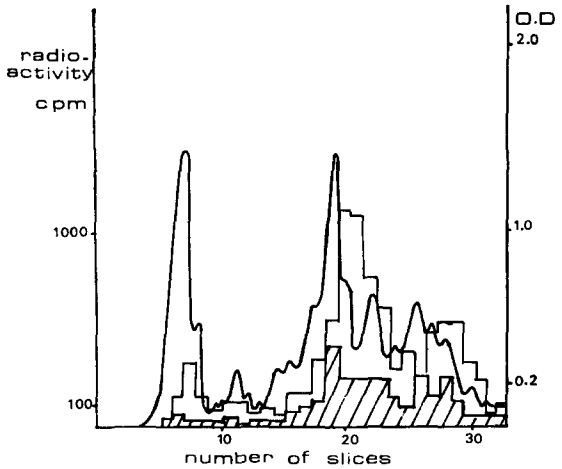

Fig. 3. Distribution of [^{14}C] ethacrynic acid in mitochondrial proteins separated by SDS polyacrylamide gel electrophoresis; influence of substrate. Mitochondria (5 mg of proteins) were preincubated with [^{14}C] ethacrynic acid (40 nmol/mg of proteins) for 10 min in 1 ml of the standard medium with or without 10 mM succinate. 100 μg of [^{14}C] ethacrynic acid-labelled proteins were applied to the gels. Dashed part of the diagram represents [^{14}C]ethacrynic acid binding after preincubation without substrate, clear part of the diagram corresponds to [^{14}C]ethacrynic acid binding after preincubation with 10 mM succinate. The curve represents absorption measurements at 660 nm of proteins stained with Coomassie Blue, using the gel scanner ISCO model 658.

TABLE I

INFLUENCE OF ENERGETIC STATE OF MITOCHONDRIA ON NEM INHIBITION OF SWELLING IN AMMONIUM PHOSPHATE AND ON [^{3}H]NEM INCORPORATION INTO RAT LIVER MITOCHONDRIA

For experimental conditions see Materials and Methods. Number in brackets indicate the number of assays.

	No substrate	10 mM succinate	10 mM β-hydroxybutyrate
Inhibition of swelling (%)	33	38	25
Bound [^{3}H]NEM nmol/mg^{-1} of proteins	12.9±0.9 (2)	19.7±1.0 (2)	15.8±0.4 (2)

Comparison of ethacrynate inhibition of swelling and ethacrynate incorporation: influence of the time of preincubation, the nature and the concentration of substrate

Fig. 4A shows that inhibition of swelling by ethacrynate, even in the presence of substrate, requires approximately 10 min preincubation to be maximum, while with NEM, maximum inhibition is obtained after only 2 min.

The kinetics of ethacrynate incorporation (Fig. 4B) show that after a rapid (1–2 minutes) and substrate-independent incorporation, the presence of substrate induces an extra incorporation which reaches a maximum of about 5 nmol · mg^{-1} of proteins after 7 min incubation.

Fig. 5A illustrates that inhibition of swelling by ethacrynate depends on the

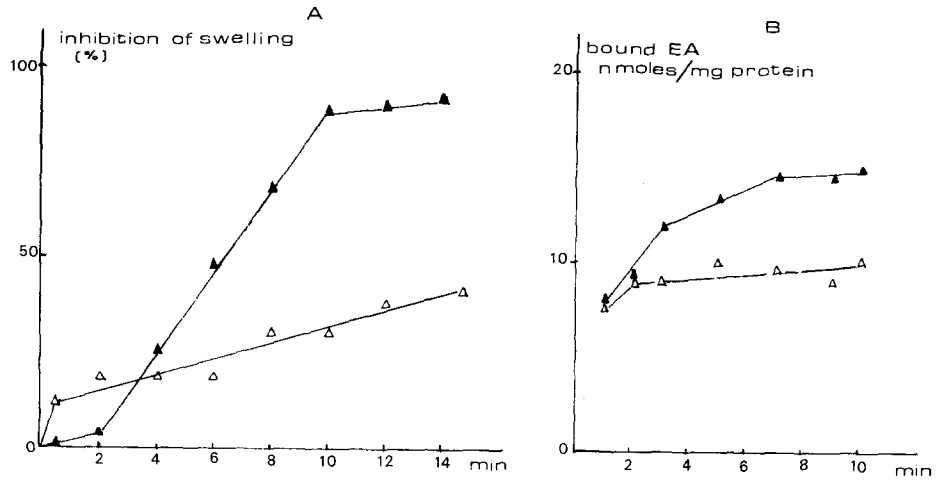

Fig. 4. Influence of time of preincubation on inhibition of osmotic swelling by ethacrynate (A) and on [^{14}C]ethacrynic acid incorporation into rat liver mitochondria (B). Mitochondria (4.25 mg of protein in A, 5.4 mg of protein in B) were preincubated with ethacrynic acid (EA) (47 nmol/mg protein in A or 37 nmol/mg protein in B) for the time indicated without substrate (△ — △) or in the presence of 10 mM succinate (▲ — ▲).

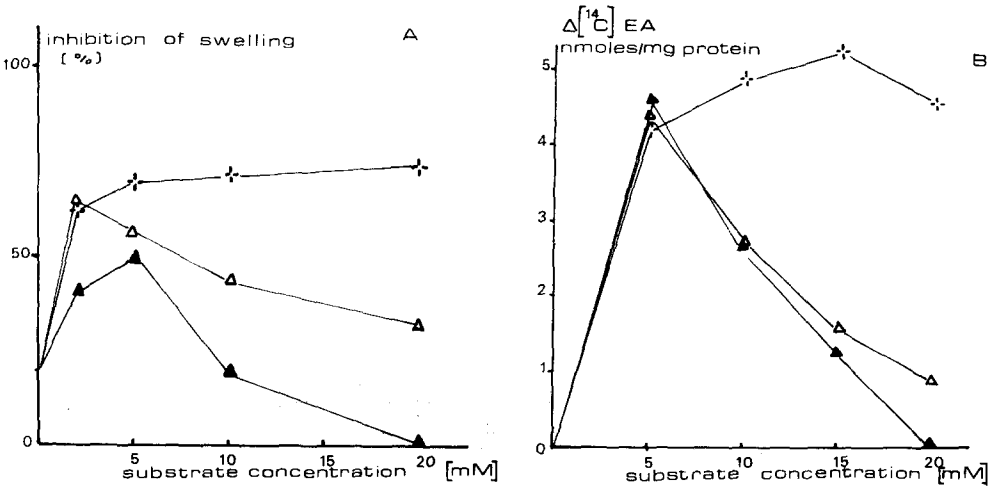

Fig. 5. Influence of the nature and the concentration of substrate on: A, ethacrynate inhibition of swelling in ammonium phosphate; B, [^{14}C]ethacrynate incorporation into rat liver mitochondria. Mitochondria (5 mg of proteins) were preincubated with 40 nmol ethacrynic acid mg^{-1} of proteins for 10 min in 1 ml of the standard medium, in the presence of different concentrations of succinate (⊹ — ⊹), glutamate (△ — △) or β-hydroxybutyrate (▲ — ▲). Osmotic swelling and bound ethacrynic acid were estimated as described under Materials and Methods. In B, Δ[^{14}C]EA (ethacrynic acid) is the difference between [^{14}C]EA binding measured with and without substrate.

nature and concentration of the substrate. With succinate, maximum inhibition is obtained for approx. 5 mM succinate and is unaffected for higher succinate concentrations. With glutamate and β-hydroxybutyrate, after a maximum inhibition obtained with 2 mM glutamate or 5 mM β-hydroxybutyrate, inhibition decreases with higher substrate concentration; this last effect is quite important with β-hydroxybutyrate, since at a concentration of 20 mM, inhibition is completely released.

Fig. 5B shows a good correlation between the substrate-effect on inhibition of swelling by ethacrynate and on ethacrynate incorporation into rat liver mitochondria.

While, with succinate, the extra incorporation of ethacrynate into rat liver mitochondria is kept even for the highest succinate concentrations used, with NAD-linked substrates, after the stimulated incorporation obtained for low substrate concentrations, there is a decrease of ethacrynate incorporation; for the highest substrate concentrations used, the amount of ethacrynate incorporated is nearly equal to the amount measured in the absence of substrate.

Influence of electron transfer inhibitors and uncoupling agents on swelling inhibition by ethacrynate

With succinate (Table II) antimycin completely cancels the stimulating effect of substrate on ethacrynate inhibition. Similar results were obtained by adding rotenone to the medium containing NAD-linked substrates.

Moreover, non-substrate dicarboxylates (malonate or *n*-butylmalonate) have no effect on the ethacrynate inhibition.

The presence of an uncoupling agent (CCCP) completely cancels the stimulated inhibition whatever the substrate is.

On the other hand, NEM inhibition of swelling in ammonium phosphate is insensitive to electron transfer inhibitors and uncoupling agents.

Influence of inorganic phosphate in the presence of different substrates

With the three substrates studied, the addition of 1 mM P_i during preincubation decreases the inhibition of swelling by ethacrynate (Fig. 6).

For any concentration of succinate inhibition is almost completely released, while without substrate, P_i has no effect.

With 5 mM glutamate there is approximately 60 % release of inhibition and with 10 mM glutamate the inhibition is completely abolished.

TABLE II

INFLUENCE OF THE ENERGETIC STATE OF MITOCHONDRIA ON ETHACRYNATE OR NEM INHIBITION OF SWELLING IN AMMONIUM PHOSPHATE

For experimental conditions see Material and Methods. Ethacrynic acid: 40 nmol · mg^{-1} of proteins; NEM: 11 nmol · mg^{-1} of proteins.

		No substrate	10 mM succinate	10 mM succinate + 10 μg antimycin	10 mM succinate + 1.25 μM CCCP
Inhibition of osmotic swelling (%)	Ethacrynic acid	21	73	12	23
	NEM	33	38	40	42

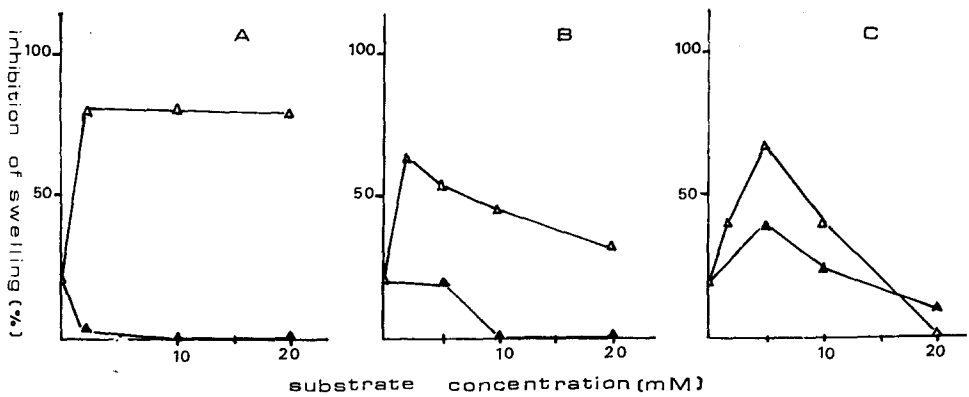

Fig. 6. Influence of P_i on ethacrynate inhibition of swelling with rat liver mitochondria in ammonium phosphate measured in the presence of different substrates at varying concentrations. Mitochondria (5 mg) were preincubated with ethacrynic acid (40 nmol · mg^{-1} of protein) for 10 min in the presence of succinate (A), glutamate (B) or β-hydroxybutyrate (C) at different concentrations. For each substrate, essays were performed in the absence (△ — △) or in the presence (▲ — ▲) of 1 mM P_i.

With β-hydroxybutyrate, the decrease of inhibition is lower than with the other substrates and there is never complete release of inhibition whatever the β-hydroxybutyrate concentration is.

Influence of Mg^{2+}

As shown in Table III, Mg^{2+} is required for significant stimulation by succinate of swelling inhibition by ethacrynate. On the other hand, Mg^{2+} is needed for stimulation by succinate of [^{14}C]ethacrynate incorporation into mitochondria.

TABLE III

INFLUENCE OF Mg^{2+} ON ETHACRYNATE INHIBITION OF SWELLING WITH RAT LIVER MITOCHONDRIA IN AMMONIUM PHOSPHATE AND ON [^{14}C]ETHACRYNATE INCORPORATION

For experimental conditions, see Materials and Methods. Figures in brackets indicate the number of assays.

$MgCl_2$ (mM)	Inhibition of osmotic swelling (%)		Bound [^{14}C]ethacrynic acid nmol/mg of protein	
	No substrate	10 mM succinate	No substrate	10 mM succinate
0	38	28	9.4±0.5 (6)	9.9±0.6 (4)
4	38	45	11.0±0.7 (5)	14.9±0.3 (4)
8	41	77	10.6±0.6 (4)	15.9±0.9 (4)

DISCUSSION

The reported results suggest that the reactivity of the P_i carrier -SH groups is influenced by the energetic state of mitochondria.

The following experimental arguments agree with this assumption: (a), the substrate-stimulated inhibition of swelling obtained with ethacrynate is due to the reactivity of ethacrynate towards thiol groups, since its saturated homologue, dihydroethacrynate (unreactive with thiol groups) never inhibits the swelling, whatever the conditions are. (b), The stimulation of swelling inhibition occurs when mitochondria are incubated with an oxidizable substrate (malonate or *n*-butymalonate have no effect) and is cancelled by inhibitors of electron transfer or by uncoupling agents. However, it must be emphasized that added ATP (without substrate) does not induce this effect and that with NAD-linked substrates the stimulation is obtained with low substrate concentrations, while the inhibition is more or less released by higher concentrations.

We assume that the increase of ethacrynate incorporation into rat liver mitochondria following substrate-induced energization [13, 14] is responsible for the observed inhibition of swelling of rat liver mitochondria by ethacrynate. This assumption is supported by the fact that ethacrynate binding and phosphate transport inhibition by ethacrynate exhibit the same characteristics: requirement of an oxidizable substrate, sensitivity to electron transfer inhibitors, to uncoupling agents, to P_i and to Mg^{2+}.

By comparing ethacrynate and NEM bindings into rat liver mitochondria [14], we came to the conclusion that the increase of binding of these two thiol reagents induced by coupled oxidation reflects an increase of reactivity or accessibility of SH groups. This conclusion is further substantiated by the electrophoresis analysis of mitochondrial proteins presented in this paper.

The release of ethacrynate inhibition of swelling by P_i is in agreement with previous data obtained with other -SH reagents [9–11, 19]; moreover, we have shown that several ethacrynate-induced effects on mitochondria are P_i-sensitive [20, 21] and that [^{14}C]ethacrynate incorporation into rat liver mitochondria is decreased in the presence of P_i [13, 14].

The compulsory presence of Mg^{2+} to obtain substrate-stimulated inhibition of swelling by ethacrynate may be related to the observed stimulation of ethacrynate incorporation by Mg^{2+} in the presence of substrate.

The decrease of ethacrynate inhibitory effect on swelling observed with the highest concentrations of NAD-linked substrates used can be explained by the decrease of ethacrynate incorporation observed under the same conditions. This decrease of ethacrynate binding needs further investigation in order to be explained.

The increase of inhibition of swelling by ethacrynate when mitochondria are energized may imply that there are two different types of SH groups related to the P_i transport: one which is accessible to ethacrynate whatever the energetic state is, the other being accessible only when mitochondria are energized.

The fact that with NEM, contrary to ethacrynate, no energy-dependent inhibition effect on the P_i carrier could be obtained may be due to the different reactivity or accessibility of the SH groups towards these two SH reagents. We assume that NEM, being far more reactive, would react almost immediately with the two types of SH groups we postulate.

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