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INFLUENCE OF THE ENERGETIC STATE OF MITOCHONDRIA ON THE INHIBITION OF OXIDATIVE PHOSPHORYLATION BY *N*-ETHYLMALEIMIDE

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Summary

N-Ethylmaleimide inhibitory effect on oxidative phosphorylation, adenylic nucleotide translocation, succinate dehydrogenase and succinoxidase activities was studied as a function of the energetic state of mitochondria.

1. Using a reversible thiol reagent (mersalyl), in order to protect the phosphate carrier against irreversible action of *N*-ethylmaleimide, it was found that: (a) when mersalyl-pretreated mitochondria were in a 'non-energized' state, i.e. preincubated without a substrate and in the presence of rotenone, only a slight inhibition of succinate oxidation coupled to ATP synthesis by *N*-ethylmaleimide was observed. (b) when mersalyl-pretreated mitochondria were in an 'energized' state, i.e. preincubated in the presence of an oxidizable substrate, *N*-ethylmaleimide strongly inhibited the coupled oxidation of succinate.

2. Mitochondrial energization was also shown to enhance the inhibitory effect of *N*-ethylmaleimide on adenylic nucleotide translocation and succinoxidase activity. However, other sulphydryl groups seem to be involved in the inhibition mechanism, but their function is unknown.

3. As *N*-ethylmaleimide inhibitory effect increased, an enhancement of *N*-[^{14}C]ethylmaleimide binding to mitochondrial sulphydryl groups was obtained.

Introduction

The involvement of sulphydryl groups in some important transport systems is well documented: phosphate [1—5], adenylic nucleotides [6,7], Krebs cycle

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid.

intermediates [8—10]. Moreover, some partial reactions of the oxidative phosphorylation are inhibited by thiol reagent: the uncoupler-stimulated ATPase [11], the P_i-ATP exchange reaction [5,12,13]. Several authors are in favour of a participation of sulphydryl groups in the coupling mechanism [14—16].

It was established by colorimetric estimation [17,18] and by labelling with radioactive compounds [19] that the reactivity and/or the accessibility of some mitochondrial sulphydryl groups towards some thiol reagents, were dependent on the energetic state of mitochondria.

The present study is an attempt to characterise sulphydryl groups linked with the oxidative phosphorylation 'machinery', whose reactivity (or accessibility) is modified by energization of mitochondria. For this purpose, we studied the effect of *N*-ethylmaleimide on succinate oxidation coupled to ATP synthesis and on some other mitochondrial reactions as a function of the energetic state of mitochondria.

N-Ethylmaleimide is a potent inhibitor of phosphate transport [3] and thus inhibits all the phosphate-dependent reactions in mitochondria [5]. However, the phosphate carrier can be protected from *N*-ethylmaleimide inactivation by pretreatment of mitochondria with a reversible thiol reagent [20]. Using this technique, we were able to study the inhibitory effects of *N*-ethylmaleimide on oxidative phosphorylation, excluding its action on the phosphate carrier.

It appears from our results, that the efficiency of *N*-ethylmaleimide as an inhibitor of oxidative phosphorylation was strongly potentiated by energisation of mitochondria.

When mitochondria were 'non-energized', inhibition of oxidative phosphorylation by *N*-ethylmaleimide was mainly due to the inactivation of the phosphate carrier. However, in these conditions, succinoxidase was also partially inhibited.

Besides the inhibition of phosphate transport, which was previously shown to be independent of the mitochondrial energy state [21], we have shown that the energization of mitochondria induced by substrate oxidation (succinate or β-hydroxybutyrate), enhanced the reaction of *N*-ethylmaleimide with the sulphydryl groups of ADP-ATP translocase. Quite unexpectedly, the glutamate oxidation did not induce this reaction. The inhibition of succinoxidase by *N*-ethylmaleimide was also slightly increased by energization. The results indicate that sulphydryl groups other than those belonging to the phosphate carrier, the adenylic nucleotide carrier, or the succinoxidase system should be involved in the inhibitory process, especially when energization was achieved by the oxidation of a NAD^+-linked substrate.

Materials and Methods

Rat liver mitochondra were prepared according to Weinbach [22]; mitochondrial proteins were estimated by the biuret method, using 0.2% cholate as solubilizing agent [23].

Oxygen uptake measurements were performed in the presence of the respiratory medium containing 62 mM sucrose, 8 mM $MgCl_2$, 50 mM KCl, 20 mM HEPES (pH 7.3) and supplemented with 4 mM succinate and 4 mM phosphate. State 3 was induced by adding 0.8 μmol ADP. Osmotic swelling of mitochon-

dria suspended in ammonium salt was measured using 1.9 ml 100 mM ammonium phosphate or 100 mM ammonium malate (plus 5 mM ammonium phosphate) plus 0.5 mM EGTA. Absorbance changes were followed at 546 nm.

For the assay of ADP translocation, mitochondria were preincubated with 0.75 mM *N*-ethylmaleimide and 1.5 mM dithiothreitol as described in the legends. 3 min after dithiothreitol addition, 20 μl 20 mM [^{14}C]ADP (specific activity: 80—100 dpm/nmol) was added; 30 s later, the reaction was stopped by 0.1 mM sodium atractylate. The samples were immediately centrifuged for 3 min; the pellets were rinsed twice with the incubation medium and solubilized with formic acid before counting in a PPO/POPOP/toluene scintillation fluid in the presence of ethanol (10 : 4, v/v). Correction for ADP trapped outside the matrix was made from the value of ADP incorporation which was obtained in experiments where atractyloside was added before [^{14}C]ADP.

Preparation of mitochondrial fragments by Lubrol treatment was performed by adding 0.12 mg Lubrol/mg protein to the mitochondria; the mixture was incubated for 10 min at 30°C. A 50 μl aliquot (approx. 0.25 mg protein) was used to measure succinate dehydrogenase activity spectrophotometrically at 623 nm in the presence of phenazine methosulphate and dichlorophenolindophenol [24]. Succinoxidase activity was measured oxypolarographically [25] using 2.5 mg protein.

N-Ethylmaleimide incorporation into mitochondria was estimated using *N*-[^{14}C]ethylmaleimide instead of unlabeled *N*-ethylmaleimide. Mitochondria were incubated with 0.75 mM *N*-[^{14}C]ethylmaleimide. After 4 min, 1.5 mM dithiothreitol was added. 3 min after dithiothreitol addition, mitochondria were spun down, the pellet and centrifuge tube wall were carefully rinsed with the incubation medium and treated for counting as described for ADP translocation measurements.

Results and Discussion

For experiments reported in this paper, we shall define two different mitochondrial energy states: (a) a 'non-energized' state: when mitochondria were preincubated in the presence of rotenone and in the absence of substrate; (b) an 'energized' state: when mitochondria were preincubated with an oxidizable substrate.

Effect of N-ethylmaleimide on succinate oxidation coupled with ATP synthesis and correlation with phosphate transport inhibition

When 0.25 mM *N*-ethylmaleimide was preincubated with 'non-energized' mitochondria (Fig. 1A, curve b) no effect on state 4 oxidation was observed, but state 3 oxidation was inhibited. However, when mitochondria were pretreated with a low amount of mersalyl (0.4 nmol/mg protein) before *N*-ethylmaleimide addition and subsequently treated with an excess of dithiothreitol, in order to reverse mersalyl action, both succinate oxidation (Fig. 1A, curve c) and phosphate transport (Fig. 1B, curve c) were partly protected against inhibition. Phosphate transport, although partially inhibited in these conditions, was not the rate-limiting step in state 3 oxidation, since pretreatment with 2 nmol mersalyl/mg protein (instead of 0.4 nmol) completely protected the phosphate

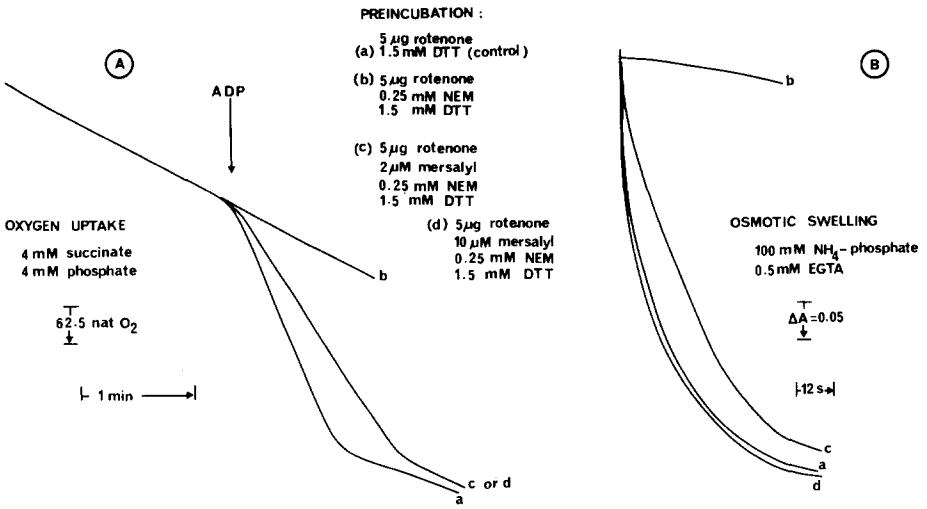

Fig. 1. Correlation between the effects of 0.25 mM *N*-ethylmaleimide on succinate oxidation coupled to ATP synthesis (A) and phosphate transport (B) after preincubation with 'non-energized' mitochondria. Influence of mersalyl pretreatment. 5 mg mitochondrial protein were incubated for 1 min in 1 ml respiratory medium (pH 7.3) supplemented with 5 µg rotenone. SH-reagents, when present, were added as follows: mersalyl, 0.4 nmol/mg protein (2 µM) (A, curve c; B, curve c) or 2 nmol/mg protein (10 µM) (A and B, curve d) 1 min after mitochondria; 0.25 mM *N*-ethylmaleimide (NEM) (A, curves b, c and d; B, curves b, c and d) 1 min after mersalyl. In all experiments 1.5 mM dithiothreitol (DTT) was added in the medium 6 min after mitochondria.

carrier (Fig. 1B, curve d) but did not give a better protection on succinate oxidation (Fig. 1A, curve d).

Fig. 2A shows that *N*-ethylmaleimide, in a concentration range varying from 0.1 to 1.0 mM, had no inhibitory effect on state 4 oxidation when preincuba-

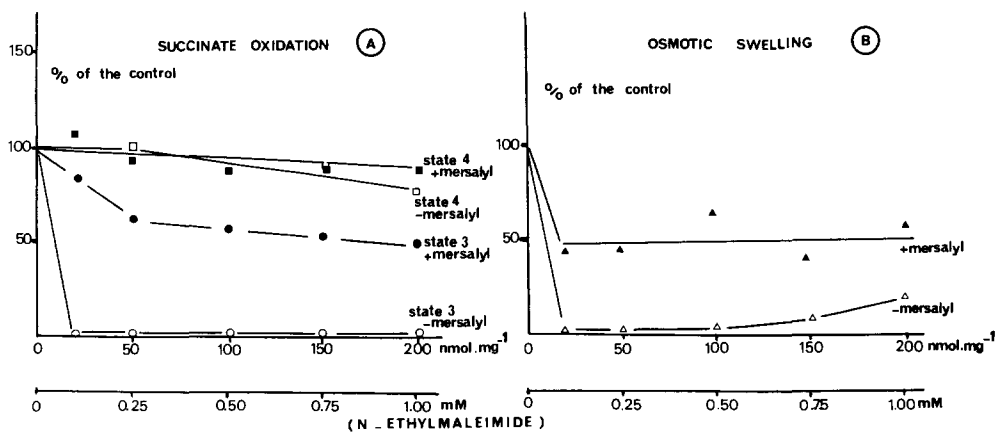

Fig. 2. Effects of different N-ethylmaleimide concentrations on state 4 and state 3 succinate oxidations (A), on phosphate transport (B) after preincubation with 'non-energized' mitochondria. Influence of pretreatment with mersalyl. 5 mg protein were incubated for 1 min in 1 ml respiratory medium plus 5 µg rotenone. Mersalyl (0.4 nmol/mg protein), when present, was added 1 min after mitochondria, followed 1 min later by *N*-ethylmaleimide. In all experiments, 1.5 mM dithiothreitol was added in the medium 6 min after mitochondria.

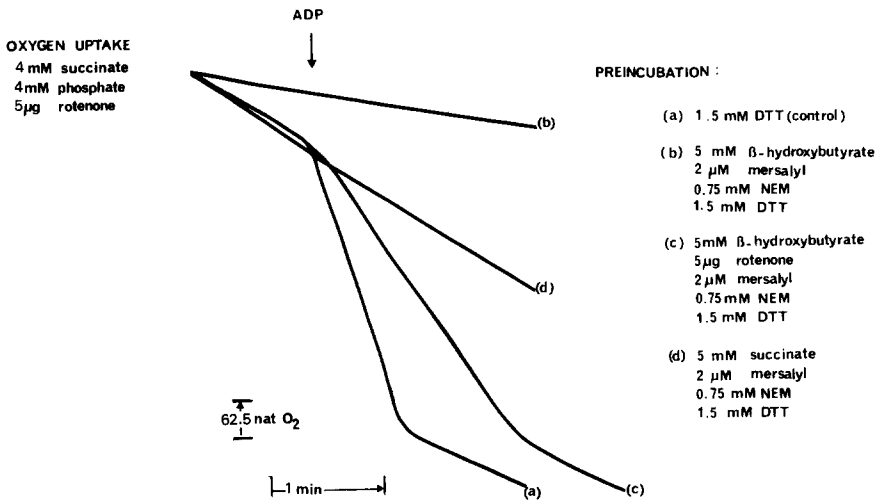

Fig. 3. Influence of energization on the effect of *N*-ethylmaleimide on succinate oxidation after preincubation with mersalyl-pretreated mitochondria. 5 mg mitochondrial protein were added to 1 ml respiratory medium (pH 7.3) containing either 5 mM β-hydroxybutyrate (curves b and c) or 5 mM succinate (curve d), 5 μg rotenone were also present in experiment c. Successive additions of the reagents were made as follows 0.4 nmol mersalyl/mg protein (2 μM): 1 min after mitochondria; 0.75 mM *N*-ethylmaleimide (NEM): 2 min after mitochondria; and 1.5 mM dithiothreitol (DTT): 6 min after mitochondria.

tion with mersalyl-pretreated mitochondria was performed in the absence of an energy supply. In the same experimental conditions, state 3 oxidation was partially maintained: 40—50% inhibition. Omission of mersalyl during preincubation gave complete inhibition of state 3 oxidation by *N*-ethylmaleimide, but state 4 oxidation was not greatly affected (Fig. 2A).

Fig. 2B illustrates parallel experiments concerning inhibition by *N*-ethylmaleimide of the osmotic swelling of mitochondria in ammonium phosphate. In agreement with other authors [20,26,27], a total inhibition of phosphate uptake by 0.1 mM *N*-ethylmaleimide was observed, but for concentration higher than 0.5 mM, the swelling was not completely suppressed. However, an equal protection of the swelling was obtained by 0.4 nmol mersalyl/mg protein against any *N*-ethylmaleimide concentrations in the range 0.1—1.0 mM.

Modifications of the energetic state of mitochondria during preincubation had drastic consequences on *N*-ethylmaleimide inhibitory potency. The presence of β-hydroxybutyrate during the preincubation of 0.75 mM *N*-ethylmaleimide with mersalyl-pretreated mitochondria, greatly increased the inhibition of succinate oxidation: both state 4 and state 3 were inhibited (Fig. 3, curve b). Similar results were obtained using glutamate instead of β-hydroxybutyrate (not shown). These substrate-stimulated inhibitions were cancelled when rotenone was present during preincubation (Fig. 3, curve c).

When energization was induced by succinate oxidation, state 3 respiration was completely inhibited by 0.75 mM *N*-ethylmaleimide, while state 4 was slightly activated (Fig. 3, curve d).

Effect of N-ethylmaleimide on ADP transport

A possible reason for the absence of respiratory control after *N*-ethyl-

TABLE I

EFFECT OF *N*-ETHYLMALEIMIDE ON [^{14}C]ADP UPTAKE IN MITOCHONDRIA INCUBATED UNDER VARIOUS CONDITIONS

Mitochondria (5 mg protein) were incubated at 30°C for 2 min in 1 ml respiratory medium (pH 7.3) without substrate or in the presence of 5 mM succinate or 5 mM β-hydroxybutyrate. Each experiment was performed either in the absence or in presence of 5 μg rotenone. 0.75 mM *N*-ethylmaleimide was added 2 min after mitochondria followed 4 min later by 1.5 mM dithiothreitol. 3 min after dithiothreitol addition, 0.4 mM [^{14}C]ADP (specific activity: 86 dpm/nmol) was rapidly mixed; ADP translocation was inhibited after 30 s by adding 0.1 mM sodium atractylate · 0.5-ml aliquots were analysed.

N-Ethylmaleimide (mM)	[^{14}C]ADP uptake (nmol/mg protein)						
	No addition		5 mM succinate		5 mM β-hydroxybutyrate		5 mM glutamate no rotenone
	No rotenone	5 μg rotenone	No rotenone	5 μg rotenone	No rotenone	5 μg rotenone	
0	10.0	6.4	7.5	7.6	3.9	5.1	7.0
0.75	7.2 (−28%)	6.7 (+5%)	0.9 (−88%)	0.9 (−85%)	0.5 (−85%)	6.4 (+25%)	4.3 (−38%)

maleimide treatment, was an impairment of ADP transport into mitochondria. Table I shows that, when *N*-ethylmaleimide was preincubated with 'non-energized' mitochondria, a poor inhibition of the nucleotide transport was obtained. The presence of succinate or β-hydroxybutyrate, during preincubation, strongly favoured the inhibitory effect of *N*-ethylmaleimide, while glutamate had no significant influence. Inhibition of ADP translocation was suppressed when oxidation of the substrate (β-hydroxybutyrate) was inhibited by rotenone. The presence of mersalyl in these experiments had no effect.

Effect of N-ethylmaleimide on dicarboxylate transport

Inhibition of succinate oxidation might be due to a decrease in the substrate permeation rate. The study of the osmotic swelling of mitochondria in an isotonic dicarboxylate salt (100 mM malate plus 5 mM P_i) after treatment with 2 nmol mersalyl/mg protein, 0.5 mM *N*-ethylmaleimide and 1.5 mM dithiothreitol, showed that the dicarboxylate carrier was poorly inhibited and that energization of mitochondria by a substrate was without effect (unreported result).

Effect of N-ethylmaleimide on succinate oxidation by mitochondrial fragments

We studied the influence of *N*-ethylmaleimide at two concentrations (0.25 and 0.75 mM) on succinoxidase and succinate dehydrogenase activities in mitochondrial fragments obtained by Lubrol WX treatment. Intact mitochondria were incubated with *N*-ethylmaleimide (in the absence of mersalyl) under different energetic conditions and then treated with Lubrol WX. It appears (Table II) that succinate dehydrogenase was partly inhibited. The presence of a substrate during preincubation slightly increased this inhibition, especially at the lowest *N*-ethylmaleimide concentration. Succinoxidase was inhibited as well and the influence of energization on the inhibitory effect was more pronounced (Table II).

TABLE II

N-ETHYLMALEIMIDE INHIBITION OF SUCCINATE DEHYDROGENASE AND SUCCINOXIDASE ACTIVITIES IN MITOCHONDRIAL FRAGMENTS OBTAINED FROM MITOCHONDRIA INCUBATED UNDER VARIOUS CONDITIONS AND TREATED BY LUBROL WX

Mitochondria (5 mg protein) were incubated for 2 min (in the absence or in the presence of 5 μg rotenone), in 1 ml respiratory medium at 30°C, *N*-ethylmaleimide was added 2 min later and 1.5 mM dithiothreitol was added 4 min after *N*-ethylmaleimide. At the end of the preincubation period, mitochondria were treated with Lubrol (0.12 mg/mg of protein) and succinate dehydrogenase and succinoxidase activities were measured as described under Materials and Methods. Numbers in brackets represent percent inhibition.

N-Ethylmaleimide (mM)	No substrate		5 mM succinate		5 mM β-hydroxybutyrate	
	No rotenone	5 μg rotenone	No rotenone	5 μg rotenone	No rotenone	5 μg rotenone
Succinate dehydrogenase activity ($\Delta A_{623nm} \cdot min^{-1}$)						
0	0.465	0.450	0.405	0.450	0.465	0.420
0.25	0.390 (—16%)	0.375 (—17%)	0.270 (—33%)	0.300 (—33%)	0.315 (—32%)	0.360 (—14%)
0.75	0.285 (—39%)	0.285 (—37%)	0.255 (—37%)	0.225 (—50%)	0.225 (—52%)	0.240 (—43%)
Succinoxidase activity (natoms $O_2 \cdot min^{-1}$)						
0	412	431	300	525	412	394
0.25	253 (—39%)	262 (—39%)	103 (—66%)	112 (—78%)	150 (—64%)	262 (—33%)
0.75	225 (—45%)	187.5 (—56%)	75 (—75%)	56 (—89%)	112 (—73%)	169 (—57%)

Influence of the energetic state of mitochondria on the binding of N-ethylmaleimide

Table III shows an energy-induced stimulation of *N*-ethylmaleimide incorporation into mitochondria, concomitant with the increase of inhibition potency. Addition of rotenone suppressed the increase of *N*-ethylmaleimide incorporation especially in the presence of NAD^+-linked substrate.

Concluding remarks

All these experimental data demonstrate that *N*-ethylmaleimide inhibitory potency is conditioned by energization of mitochondria.

It is shown that the inhibition of succinate-coupled oxidation, induced by preincubation of *N*-ethylmaleimide with 'non-energized' mitochondria, is due essentially to the inhibition of phosphate transport, since protection of the

TABLE III

BINDING OF *N*-ETHYLMALEIMIDE TO SULPHYDRYL GROUPS OF MITOCHONDRIA INCUBATED UNDER VARIOUS CONDITIONS

Mitochondria (5 mg protein) were preincubated at 30°C for 2 min in 1 ml respiratory medium (pH 7.3). 0.75 mM *N*-[^{14}C]ethylmaleimide was then added, followed 4 min later by 1.5 mM dithiothreitol. 3 min after dithiothreitol addition, 0.4-ml aliquots were centrifuged and analysed for radioactivity.

Present during preincubation	Bound *N*-[^{14}C]ethylmaleimide (nmol/mg protein)	
	No rotenone	+ 5 μg rotenone
Nil	25.8	21.2
5 mM β-hydroxybutyrate	34.0	20.7
5 mM succinate	33.9	29.2

carrier by mersalyl permits the coupled oxidation to take place. However the phosphate carrier is probably not the unique site of *N*-ethylmaleimide action: a slight inhibition of state 3 oxidation, independent of maleimide concentrations between 0.1 and 1.0 mM, persists even after treatment with mersalyl; moreover it was shown that succinoxidase activity was partially inhibited.

Energization of mitochondria by a substrate increases the *N*-ethylmaleimide inhibition on oxidative phosphorylation. It was shown that energization induced by succinate or β-hydroxybutyrate oxidation, favoured alkylation by *N*-ethylmaleimide of some sulphydryl groups belonging to the ADP-ATP translocator. Such an energy-dependent inhibition of nucleotide translocation by *N*-ethylmaleimide has already been reported by Vignais [28]. A more important inhibition of succinoxidase activity was observed when mitochondria were energized before *N*-ethylmaleimide treatment.

However, it is clear that inhibition of the adenylic nucleotide translocase or succinoxidase activities are not sufficient to explain all experimental data. For example, glutamate which enhances the *N*-ethylmaleimide inhibitory effect on succinate oxidation (state 3 and state 4) has no action on the adenylic nucleotide translocase sensitivity to *N*-ethylmaleimide. The reasons for the inhibition of succinate state 4 oxidation by *N*-ethylmaleimide when mitochondria were energized by a NAD^+-linked substrate are unknown. Sulphydryl groups, other than those mentioned above, may become reactive when mitochondria are energized; the identification of these sulphydryl groups is now being undertaken.

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