ON THE INHIBITION OF THE TRANSPORT OF INORGANIC PHOSPHATE BY *N*-ETHYLMALEIMIDE

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1. Introduction

The transport of inorganic phosphate in mitochondria is inhibited by sulphydryl-blocking reagents [1–6]. It is reported in this paper that, in intact rat-liver mitochondria preincubated with ionophores to minimize the accumulation of P_i in the matrix space, (NEM)*, enhances the uptake of added P_i by mitochondria [7,8]. This effect of NEM appears to involve binding of P_i to the mitochondrial membrane. Evidence is presented that the stimulation of P_i uptake by mitochondria induced by NEM is directly related to the inhibition of P_i transport across the mitochondrial membrane. Analogous results have been obtained with mersalyl [7–9].

2. Methods

Rat-liver mitochondria were preincubated for 10 min at 4°C in 0.25 M sucrose with the addition of $^{32}P_i$ (carrier free), washed and resuspended in cold sucrose. Before washing P_i was determined chemically [10] and the specific activity of $^{32}P_i$ was calculated by measuring the total radioactivity of 12% $HClO_4$ extracts. This specific activity was used to estimate the level of endogenous P_i which was corrected for the non-removable portion determined in parallel samples by adding 5 mM cold P_i. $^{32}P_i$ labelled mitochondria were incubated in a medium containing: 125 mM sucrose; 10 mM Tris–HCl; 5 μg oligomycin; 0.34 μg rotenone and 0.17 μg antimycin. The pH was adjusted to 7.8. At the time specified in the legends nigericin, FCCP, valinomycin $^{32}P_i$, NEM and butylmalonate were added. Incubation was carried out at 4°C directly in small centrifuge tubes in a total volume of 1ml and mitochondria separated from the suspending medium by rapid centrifugation at 20 000 *g*. The new specific activity was determined by taking into account the endogenous P_i and the total P_i content of mitochondrial pellet was corrected for that present in the [^{14}C] sucrose space.

3. Results

The experiment of fig. 1 illustrates the effect of NEM on the level of mitochondrial P_i. Mitochondria were incubated with added P_i in a K^+-free medium. Once the equilibrium of P_i distribution between the internal and external compartment was reached, NEM was added. Under these conditions the inhibitor had no effect on the P_i level. However when the mitochondria level of P_i was greatly reduced by preincubation with nigericin, NEM caused 100% increase of the mitochondrial P_i. Fig. 1 shows also that NEM inhibited P_i-uptake when added before the anion but, also in these conditions, increased the P_i level of mitochondria pretreated with nigericin. Table 1 shows that NEM increased the $P_i$level of mitochondria pretreated with nigericin. Table 1 shows that NEM increased the uptake of P_i also by mitochondria preincubated with an uncoupler or an uncoupler plus valinomycin.

Fig. 2 shows the existence of a direct relationship between the inhibitory effect of NEM on P_i transport and the stimulatory effect on P_i uptake so far

* *Abbreviations:* NEM, *N*-ethylmaleimide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

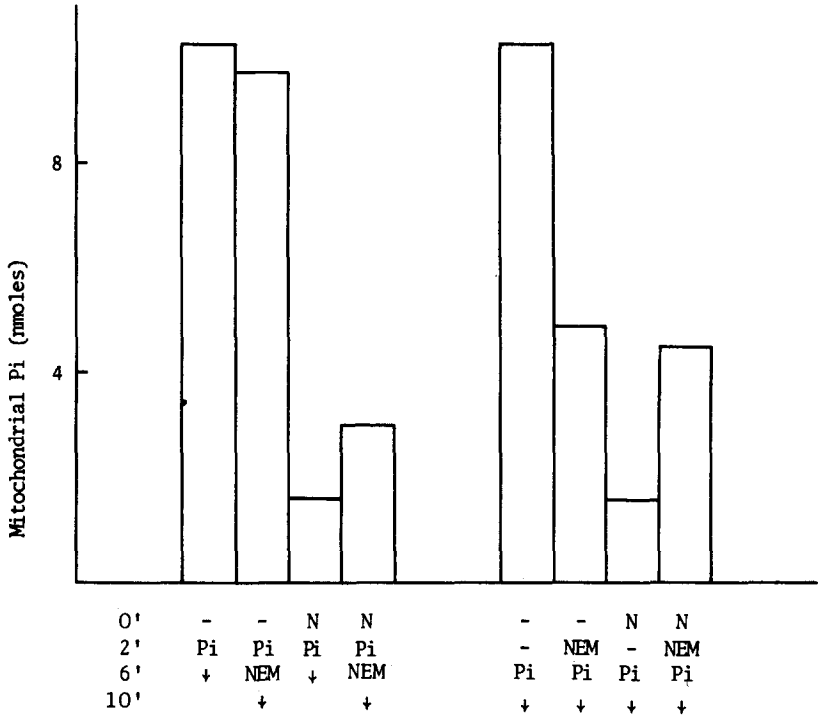

Fig. 1. Effect of NEM on mitochondrial P_i level in presence and absence of nigericin. Mitochondria (1 mg/ml) were incubated in standard medium as described. The following additions were done at the time (min) indicated: 200 μM $^{32}P_i$ (P_i), 0.14 μM nigericin (N) and 150 μM NEM. The reaction was stopped at the time indicated by the arrows.

Table 1

Effect of *N*-ethylmaleimide on mitochondrial P_i level in presence of nigericin, FCCP and valinomycin.

Additions	P_i (nmoles)	ΔP_i (nmoles)	Stimulation (%)
Nigericin	0.64		
Nigericin + NEM	1.28	+ 0.64	100
FCCP	1.29		
FCCP + NEM	2.98	+ 1.69	131
FCCP + valinomycin	0.28		
FCCP + valinomycin + NEM	0.50	+ 0.22	78

Mitochondria (1 mg/ml) were incubated in standard medium as described. Sequence of additions: zero time, 0.14 μM nigericin or 0.1 μM FCCP or FCCP plus 0.2 μg valinomycin; at 2 min, 100 μM $^{32}P_i$; at 6 min, 150 μM NEM and reaction stopped at 10 min.

described. The inhibitory action of NEM on nigericin induced efflux of P_i from mitochondria and the enhancement of the mitochondrial level of P_i caused by the reagent when added under equilibrium conditions to nigericin pretreated mitochondria, presented the same concentration dependence.

The experiments reported in figs. 3 and 4 illustrate respectively the effect of butylmalonate on $malate_{in}$–$P_{i\,out}$ exchange reaction and on enhancement of P_i level induced by NEM. At 5 μM butylmalonate gave half-maximal inhibition of the malate–P_i exchange (fig. 3) and removed (fig. 4,a) or prevented (fig. 4, b) by 50% the increase of the mitochondrial P_i level induced by NEM.

4. Discussion

The transport of P_i consists basically of two steps: the binding to the specific sites of the transporting

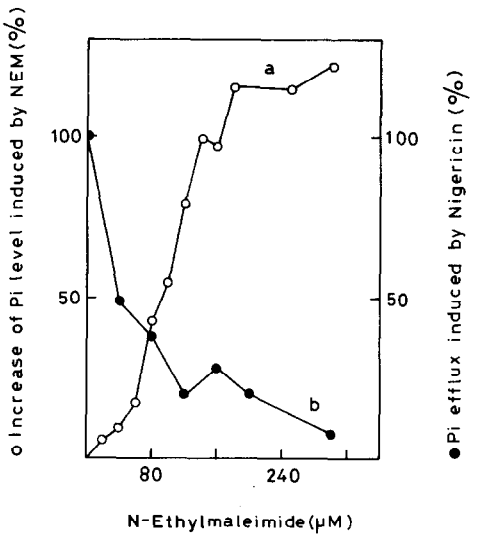

Fig. 2. Effect of NEM on mitochondrial P_i level and on P_i efflux from mitochondria induced by nigericin. Mitochondria (1 mg/1 ml) were incubated in standard medium as described. Sequence of additions in curve (a): zero time, 0.14 μM nigericin; at 2 min, 200 μM $^{32}P_i$; at 6 min, NEM as indicated and reaction stopped at 10 min. In curve (b) the sequence of additions was as in curve (a) with the difference that nigericin was added at 10 min and reaction stopped at 12 min. In the absence of NEM the P_i efflux induced by nigericin (curve, b) amounted to 10.3 nmoles and the P_i level (curve; a) to 1.9 nmoles.

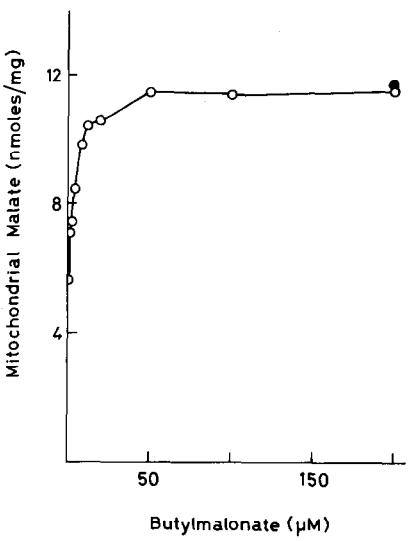

Fig. 3. Effect of butylmalonate on malate efflux induced by external P_i. Freshly isolated mitochondria were preincubated 10 min at 4°C in 250 mM sucrose with 2mM malate, washed and resuspended in cold 250 mM sucrose containing 10 μCi of [^{14}C]malate (carrier free). Loaded mitochondria (2.9 mg/ml) were incubated in standard medium as described. Malate was also determined enzymatically [11]. Sequence of additions: zero time, none; at 2 min, butylmalonate as indicated; at 4 min, 200 μM cold P_i and reaction stopped at 8 min. •, Control in the absence of P_i.

system(s) and the net movement across the membrane. Since intact mitochondria accumulate, also in the presence of metabolic inhibitors, a large amount of externally added P_i, it is difficult to distinguish between P_i free inside the matrix and that bound to the membrane. In the present investigation accumulation of P_i by mitochondria was minimized by preincubation with nigericin or FCCP plus valinomycin. These substances induce in a K^+-free medium an exchange between internal K^+ and external H^+ [12, 13], with the consequence that mitochondria lose the capability of maintaining a concentration gradient of P_i. Under these conditions NEM increased the mitochondrial level of P_i either if added before or after external P_i. Furthermore the stimulatory effect of NEM was removed or abolished by butylmalonate. The exact correspondence of the titer of the inhibitory effect of NEM on P_i transport and that for the enhancement of the mitochondrial P_i level as well as the high affinity of butylmalonate in counteracting NEM effect, strongly suggest that NEM stimulates the binding of P_i on specific sites of carrier system(s), and that this is related to the inhibition of P_i transport by this reagent. The stimulation could be due to a decrease of the dissociation constant of P_i from the transporting system(s) or to an increase of the number of binding sites as a consequence of carrier(s) modification induced by NEM. In the absence of nigericin the stimulatory effect of NEM on P_i binding is apparently masked by the large amount of P_i accumulated by mitochondria.

It is generally known that in mitochondria NEM inhibits specifically the P_i—OH^- exchange whilst butylmalonate inhibits the P_i—malate exchange reaction. The present results apparently favour the existence of one P_i transporting system with sites differently sensitive towards the inhibitors [see also ref. 4]. However they do not exclude the possibility of the existence of two interacting P_i-carriers.

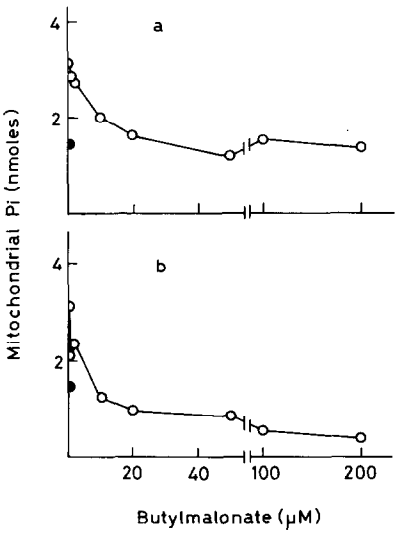

Fig. 4. Effect of butylmalonate on mitochondrial P_i level. Mitochondria (1 mg/ml) were incubated in standard medium as described. Sequence of additions: in Expt. (a): at zero time, 0.14 μM nigericin; at 2 min, 150 μM NEM; at 6 min, 200 μM $^{32}P_i$; at 10 min, butylmalonate as indicated and reaction stopped at 14 min; in Expt. (b): butylmalonate was added at 6 min and $^{32}P_i$ at 10 min. ●, Control in the absence of NEM.

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