

## FUMARATE TRANSPORT BY RAT LIVER MITOCHONDRIA

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## 1. Introduction

Evidence has been presented for the existence of carrier systems located in the inner mitochondrial membrane for the transport of adenine nucleotides [1], inorganic phosphate [2–5], Krebs cycle intermediates [6–7], amino acids [8] and pyruvate [9]. In this paper results are presented which reveal the probable existence of a carrier system for fumarate. The evidence for the fumarate carrier is, firstly that the swelling of mitochondria in  $\text{NH}_4$ -fumarate is taking place only when SH reagent and ADP (or ATP + oligomycin) is present; secondly that the swelling induced by SH reagent + ADP is diminished by 5'-AMP; and thirdly that this process is temperature dependent. It is proposed that translocation of fumarate across the inner mitochondrial membrane is directly coupled to hydroxyl ion counterflux.

## 2. Materials and methods

Rat liver mitochondria were prepared as described by Weinbach [10] in 0.25 M sucrose + 3 mM tris-HCl (pH 7.5). Protein was estimated by the biuret method [11]. Swelling was monitored at 30° by the absorbance at 546 nm in glass cuvette using an Unicam SP 800 recording spectrophotometer. The reaction mixture (final volume 3 ml) contained: 80 mM ammonium fumarate, 5 mM tris-HCl (pH 7.5), 4  $\mu\text{g}$  antimycin, 4  $\mu\text{g}$  rotenone and 3 mg of protein. Other additions were as indicated in the figures.

Fumarate was obtained from BDH; ADP from Calbiochem; DTT (dithiothreitol) was purchased from P-L Biochemicals. ATP, ITP, 5'-AMP, 3'-AMP and pCMB (*p*-chloromercuribenzoate) were produced by Koeh-Light; EDTA and oligomycin by Sigma Chemical Co., merphtallil (methoxytheophyllinmercuri-propylphthalimide) by Polfa (Poland).

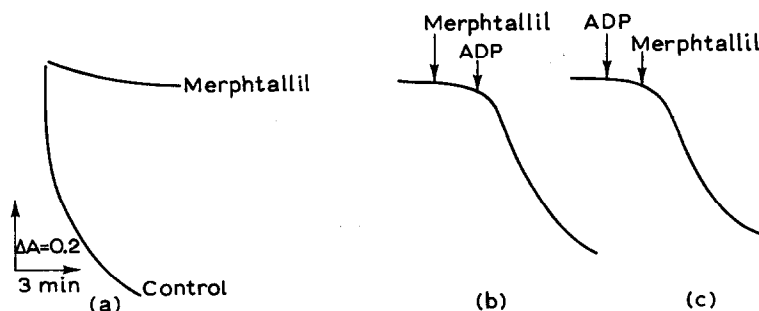
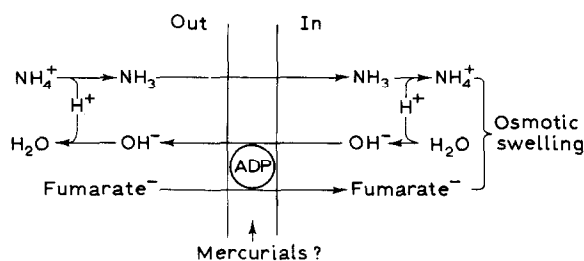


Fig. 1. Swelling of rat liver mitochondria in  $\text{NH}_4$ -phosphate (a) and in  $\text{NH}_4$ -fumarate (b, c). (a) Mitochondria (3 mg of protein) were suspended in 3 ml 80 mM  $\text{NH}_4$ -phosphate, 3 mM tris-HCl (pH 7.5), 2  $\mu\text{g}$  rotenone and 2  $\mu\text{g}$  antimycin A. (b and c) The experiments were carried out under the conditions described in Materials and methods. Where indicated ADP (0.3 mM) and merphtallil (30  $\mu\text{M}$ ) were added.



Scheme 1. Proposed model for osmotic swelling of isolated mitochondria in  $\text{NH}_4$ -fumarate in the presence of ADP and merphtallil.

### 3. Results and discussion

It has been shown that SH reagents block swelling of mitochondria in ammonium phosphate [4]. First, the effect of merphtallil on mitochondrial swelling in ammonium phosphate was studied. Fig. 1a shows that merphtallil inhibited mitochondrial swelling in ammonium phosphate. This indicates that merphtallil acts on the transport of phosphate similarly to other SH reagents. Previously, it had been observed that reagents which can be presumed to react primarily with membrane SH groups bring about major alterations in the ability of the mitochondrion to take up and retain  $\text{K}^+$  [12–14]. It had also been observed that some mercurials caused the mitochondrial membrane to become permeable to  $\text{Cl}^-$  [15, 16]. We observed that merphtallil stimulated swelling of rat liver mitochondria suspended in ammonium chloride (not shown here). As indicated in fig. 1b, merphtallil alone was without effect on mitochondrial swelling in ammonium fumarate; addition of ADP produced swelling of rat liver mitochondria suspended in  $\text{NH}_4$ -fumarate in the presence of merphtallil (fig. 1b, c). The above data indicate that swelling in  $\text{NH}_4$ -fumarate occurs only when ADP and merphtallil are present in the medium. We assumed, therefore, that in the inner mitochondrial membrane a 'latent' carrier system for fumarate is present. Some suggestions have been reported about the non-phosphorylating role of ADP [17]. According to Leblanc, ADP is specifically required for the active uptake step of calcium ion through the mitochondrial membrane [18]. Weber et al. suggested that

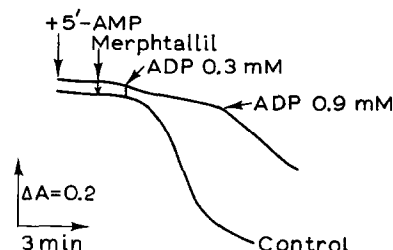


Fig. 2. The effect of 5'-AMP on mitochondrial swelling in  $\text{NH}_4$ -fumarate in the presence of ADP and merphtallil. The experiments were carried out as described in Materials and methods. Where indicated 5'-AMP (0.2 mM), merphtallil (30  $\mu\text{M}$ ) and ADP were added.

ADP induces gross morphological transition of the inner mitochondrial membrane, which probably reflects the consequences of specific binding to the latter [19]. It is possible that the carrier proposed is activated by ADP in the presence of merphtallil. It has been shown recently that some carriers require specific activator for maximal activity [6, 7]. According to Klingenberg [20], the specific binding of ADP to the inner membrane is selectively sensitive to methylene blue-catalyzed photooxidation. We examined the effect of ADP on the swelling of mitochondria in  $\text{NH}_4$ -fumarate in the presence of merphtallil after preincubation with methylene blue according to Klingenberg [20]. We observed that swelling was similar, as presented in fig. 1b. The above data indicate that the effect of ADP was not related to the binding of this nucleotide to specific sites. ADP might be replaced by ATP (+ oligomycin) but not by 5'-AMP, 3'-AMP or ITP. These results suggest specificity of the stimulatory effect of ADP and ATP on the proposed carrier system. The role of SH reagents is not clear. Similar results were observed when merphtallil was replaced by other SH reagents e.g. pCMB. As it has been previously observed, SH reagents reacting with membrane SH groups caused the mitochondria to become permeable to some anions and cations [12–16]. Brierley et al. also suggested that mercurials render the mitochondrial membrane permeable to  $\text{H}^+$  or  $\text{OH}^-$  ions [16]. It is probable that merphtallil caused a disclosure of 'latent' carrier for fumarate which requires ADP or ATP for activation. The postulated mechanism

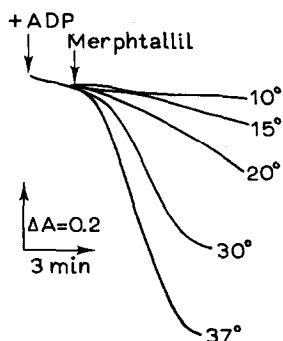


Fig. 3. Temperature dependence of swelling of rat liver mitochondria in  $\text{NH}_4$ -fumarate (+ ADP + merphthallil). Experimental conditions as described in Materials and methods.

of mitochondrial swelling in  $\text{NH}_4$ -fumarate in the presence of ADP or ATP and merphthallil is shown in scheme 1.  $\text{NH}_3$  passes into the mitochondrial membrane down a concentration gradient [21]. Once inside, the mitochondrion,  $\text{NH}_3$  associates with protons leaving an excess of  $\text{OH}^-$  which can then exchange for fumarate made diffusible by ADP and merphthallil. Evidence for a carrier for the transport of fumarate across the mitochondrial membrane is indicated by the effect of 5'-AMP on the swelling of mitochondria suspended in  $\text{NH}_4$ -fumarate in the presence of ADP and merphthallil. Fig. 2 shows that if 5'-AMP is added to the medium, mitochondria suspended in  $\text{NH}_4$ -fumarate in the presence of ADP and merphthallil do not swell. 3'-AMP was without effect. Excess ADP reversed the inhibitory effect of 5'-AMP. According to Klingenberg [20, 22, 23] carrier systems are temperature dependent. Fig. 3 shows the effect of different temperatures on mitochondrial swelling in  $\text{NH}_4$ -fumarate in the presence of ADP and merphthallil. As shown in fig. 3, the degree of mitochondrial swelling is temperature dependent. These results show that the criterion of significant temperature dependence is fulfilled by the proposed translocator for fumarate. It is known that reagents such as cysteine and dithioerythritol remove the external mercurials from the mitochondrial membrane [16]. Kurup et al. reported that the effect of mercurials on NADH oxidation in submitochondrial particles

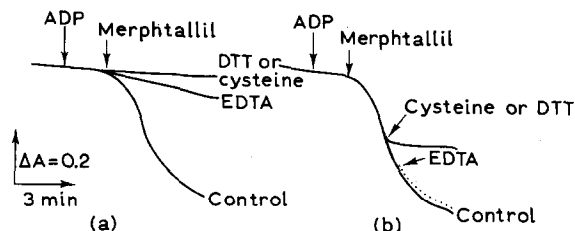


Fig. 4. The effect of EDTA, DTT or cysteine on mitochondrial swelling in  $\text{NH}_4$ -fumarate. The experiments were carried out as described in Materials and methods. EDTA (0.4 mM), DTT (0.05 mM) and cysteine (0.1 mM) were added as indicated.

was masked by EDTA [24]. Fig. 4 shows the swelling of mitochondria in  $\text{NH}_4$ -fumarate induced by merphthallil + ADP in the presence of EDTA, DTT or cysteine. In all cases mitochondria swelled only slightly. Cysteine or DTT added after merphthallil gave complete inhibition of mitochondria swelling in contrast to EDTA, which was without effect (fig. 4). Similar effects of EDTA on mercurials inhibition of NADH oxidation in submitochondrial particles have been previously observed [24].

Although fumarate has been regarded as a non-penetrant anion [21], its physiological role does not seem to be reconcilable with this motion. We were able to show penetration of fumarate into mitochondrion under the conditions described. The question arises whether the proposed mechanism can operate under physiological conditions. The answer both to this question and to the validity of the proposed mechanism will have to await further experimentation.

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