1. Introduction

In a recent exhaustive analysis of the reactivity of -SH reagents with the mitochondrial 'phosphate carrier', Klingenberg et al. [1] reported that Pi protects against inhibition by maleimide derivatives. The same authors also reported that endogenous Pi protects better than exogenous Pi. Unlike monothiol reagents (maleimides, mercurials etc.), diamide (diazenedicarboxylic acid bis dimethylamide), a thiol oxidizing agent, does not inhibit Pi transport across the mitochondrial membrane, but facilitates it [2]. Moreover, diamide induced swelling of rat liver mitochondria suspended in medium containing Pi is prevented by Mg²⁺ [3].

In the light of these observations it appeared interesting to investigate whether diamide, on one hand, and Mg²⁺, on the other, could interfere with the action of mercurials on Pi transport.

2. Experimental

Rat liver mitochondria were isolated in 0.25 M sucrose following Schneider and Hogeboom [4]. Mitochondrial protein was determined by a biuret method. ³²P⁻⁻⁻ uptake was determined as described by Papa [5]. The Pi efflux from mitochondria was followed with the swelling method, based on the generation of Pi in the mitochondrial matrix by the FCCP stimulated ATP hydrolysis as described by Klingenberg et al. [1].

For the direct measurement of Pi efflux, ³²P⁻⁻⁻ generated in the FCCP [γ³²P]ATP system was determined both in the supernatant and in the pellet following the procedure of Ernser et al. [6]. ³²P⁻⁻⁻ was counted by Packard Tri-Carb Spectrophotometer.

3. Results

As shown in fig.1 diamide did not affect mersalyl inhibition of Pi transport. Likewise diamide did not
Table 1
Action of mersalyl and Mg** on the uptake of 32 Pi by rat liver mitochondrial

<table>
<thead>
<tr>
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<th>32 Pi uptake</th>
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<tr>
<td></td>
<td>CPM/mg protein</td>
</tr>
<tr>
<td>Control</td>
<td>626</td>
</tr>
<tr>
<td>Control + Mg**</td>
<td>625</td>
</tr>
<tr>
<td>Mersalyl</td>
<td>224</td>
</tr>
<tr>
<td>Mersalyl + Mg**</td>
<td>220</td>
</tr>
</tbody>
</table>

Experimental conditions as in fig.1. When added, mersalyl was 200 μM, and Mg** 4 mM. 0.50 nmol/ml 32 Pi (70 000 cpm) were added.

Table 1 shows that Mg** did not affect mersalyl inhibition of 32Pi uptake from the medium. On the contrary, Mg** abolished mersalyl inhibition of Pi efflux from mitochondria assayed with the ‘ATP-FCCP’ system (fig.2.). This effect was also observed in the presence of higher amounts of mersalyl (up to 2 mM). Diamide was uneffective on the inhibition of mersalyl of Pi efflux (fig.2).

That the suppression of mersalyl induced swelling by Mg** was really due to the restored efflux of Pi from mitochondria and not, for instance, to the inhibition of the uncoupler stimulated ATPase activity, which generates Pi intramitochondrially, or to the precipitation of phosphate salts inside the mitochondrion, is confirmed by the results of table 2.

These results show that: 1) mersalyl, both in the presence and in the absence of Mg** did not decrease the FCCP induced ATPase activity, which, on the contrary, was increased (compare the amount of total 32Pi hydrolyzed from [γ32P]ATP); 2) in spite of the increased ATPase activity, mersalyl strongly prevented the accumulation of 32Pi in the supernatant; 3) when Mg** was present, much more 32Pi was found in the supernatant and correspondingly less in the pellet. Consequently it appears that Mg** restores mersalyl-blocked efflux of Pi by preventing, or antagonizing, the action of mersalyl, and not as a consequence of the possible above-mentioned secondary effect.

4. Discussion

The action of mersalyl, as well as other thiol reagents, on Pi transport through the inner mitochondrial membrane is generally interpreted as a consequence of an inhibition of a more or less specific ‘phosphate translocator’. [7–9] Fonyo [10] has calculated that the amount of mersalyl that causes total inhibition of phosphate translocation minus the amount of mersalyl that is not inhibitory at all, equals about 2–3 nmol/mg protein. The above reported finding that diamide does not decrease the minimal amount

Table 2
Action of mersalyl and Mg** on the efflux of 32 Pi formed inside the mitochondria from 32P ATP

<table>
<thead>
<tr>
<th></th>
<th>32 Pi cpm/mg protein</th>
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<tbody>
<tr>
<td></td>
<td>Supernatant</td>
</tr>
<tr>
<td>Control</td>
<td>2080</td>
</tr>
<tr>
<td>+ Mersalyl</td>
<td>1433</td>
</tr>
<tr>
<td>+ Mersalyl + Mg**</td>
<td>2785</td>
</tr>
</tbody>
</table>

Rat liver mitochondria (2.5 mg/ml) were incubated 5 min at 20°C in the medium described in fig. 2 containing 2.1 μmol/ml [γ32P]ATP (49 000 cpm). When present mersalyl was 200 μM, Mg** 4 mM.
of mersalyl required for the complete inhibition of $P_i$ transport (fig.1) indicates that the population of thiols affected by diamide is not related to the thiols involved in the $P_i$ transport. In a parallel study (manuscript in preparation), it has been found that, at the concentrations used in the present work, diamide oxidizes 25% of the total mitochondrial thiols. These oxidized thiols, in the light of diamide properties [11], should include all the solubilized thiols and pairs of membrane bound thiols, sterically accessible to diamide interaction. This observation is in full agreement with the previous one that diamide, per se, does not inhibit, but rather facilitates, $P_i$ transport [2].

The observation that $Mg^{++}$ prevents the inhibition by mersalyl of $P_i$ efflux from mitochondria is in apparent contrast with the lack of effect on the inhibition by mersalyl on $P_i$ influx (compare table 2 and 1). The interpretation of this finding is very difficult also in consideration of the circumstance that, unlike heart mitochondria, liver mitochondria do not seem capable of accumulating $Mg^{++}$ [12]. On the other hand, data reported in table 2 rule out the possibility of a precipitation of $Mg^{++}$ phosphate salts inside the mitochondria. For the time being, asymmetry of the mitochondrial membrane with respect to the steric position or to the function of the thiol groups involved in the phosphate translocation appears the most plausible hypothesis.

References