Energy-linked Adenosine Diphosphate Accumulation by Corn Mitochondria

II. PHOSPHATE AND DIVERENT CATION REQUIREMENT

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ABSTRACT

The requirement for phosphate and Mg2+ in energy-linked [3H]ADP accumulation by corn mitochondria has been studied. Arsenate will fully substitute for phosphate; sulfate partially substitutes; acetate, bicarbonate, and pyrophosphate are ineffective. Phosphate is also taken up by the mitochondria, but the ADP/Pi ratio varies widely with experimental treatments. ADP does not exchange with endogenous labeled phosphate, although Pi/2Pi exchange occurs.

Mg2+ is also accumulated during ADP uptake. Mg2+ can be substituted with varying efficiency by other divalent cations, but not monovalent cations. Effective cations typically increase phosphate uptake, particularly Ca++. Ca++-activated ADP accumulation is insensitive to carboxyatractyloside over a wide range of Ca++ concentrations. When Ca++ is substituted for Mg2+, it is not necessary to block ATP formation to secure high levels of ADP accumulation, since Ca++ will divert energy from ATP formation into ion uptake.

It is suggested that the transport mechanism may carry out a concerted transport of ADP and phosphate with bound divalent cation. The phosphate transporter may be involved, or alternatively a special mechanism for trivalent anion transport may exist which acts cooperatively with the phosphate transporter.

The Millipore filtration procedure for determining [3H]ADP uptake was as described (1). The same procedure was used for 32Pi uptake. Millipore filters were presoaked in unlabeled 2 mM ADP and/or 5 mM Pi to reduce the radioactivity absorbed by the filter from the medium.

For determining if accumulated phosphate would exchange for ADP, the mitochondria were preloaded with 32Pi by incubation at room temperature for 11 min with about 12 mg protein in 9 ml of the basic medium containing 10.7 mM K, 15 mg oligomycin, 60 μmol NADH, and 4 mM MgCl2. The mitochondria were collected by rapid centrifugation of 0.8-ml aliquots in an Eppendorf 5412 centriuge; the pellets were rinsed with cold 0.2 M sucrose, and then resuspended in 0.8 ml basic medium in the centrifuge tubes containing oligomycin, NADH, and MgCl2 at the same concentrations used during loading. Additions were made as indicated in Figure 2, and after 3-min incubation the mitochondria were collected by rapid centrifugation. The final pellets were dissolved in formic acid and counted as previously reported (18).

The Mg2+ content of mitochondria was determined by using a Jarrell-Ash model 82-700 atomic absorption spectrometer. Extracts of mitochondria were collected in 12% trichloroacetic acid by using the silicone oil method as described (3). Final aliquots, where Mg2+ was measured, had a final concentration of 6% trichloroacetic acid and 0.8% SrCl2.

Radioactivity was measured in a Beckman LS-230 scintillation counter (1). Mitochondrial protein was determined by the method of Lowry et al. (15), using BSA (fraction V) as a standard. [3H]ADP and 32Pi were purchased from New England Nuclear, C-ATR from Boehringer, nucleotides, Tes, and BSA from Sigma, and all other reagents were analytical grade.

RESULTS

Phosphate and ADP Accumulation. A time course for [3H]ADP and 32Pi uptake is given in Figure 1. Compared with [3H]ADP, very little 32Pi enters the mitochondria during the nonenergized 1st min of reaction. Initial passive uptake of a small amount of [3H]ADP was observed previously (1); it has the same Km as active uptake (3). After the addition of NADH there was rapid 32Pi uptake in approximately a 1:1 ratio with ADP. However, 32Pi uptake was not changed by omission of ADP from the medium, and thus there is no indication that ADP influences the uptake of phosphate or that the ratio has any special significance. Experiments done later with a new lot of corn seed gave different ratios (Table 1).

Table I shows the response to inhibitors (mersalyl and N-ethylmaleimide) and a promoter (roteneone) of phosphate transport, which also act on ADP uptake (3). In all cases the ADP/Pi uptake ratio was lowered from the control value of 0.77. Evidently, ADP uptake is linked in some fashion with phosphate uptake, but there is no fixed stoichiometry to the process.

In the preceding papers (1-3) we have described the general properties of ADP accumulation by corn mitochondria. The transport mechanism is insensitive to C-ATR, the inhibitor of the ADP translocase, but is very sensitive to inhibitors of phosphate transport. Phosphate and Mg2+ are required for ADP accumulation, and questions arise as to the nature of this requirement. We report here on the specificity of the transport system for these ions, on their accumulation during ADP uptake, and on the possibility that ADP enters in exchange for phosphate. We speculate that the mechanism of ADP accumulation in corn mitochondria is one of concerted phosphate + divalent cation + ADP uptake.

MATERIALS AND METHODS

Mitochondria were isolated from 3.5-day-old etiolated corn shoots (Zea mays L.) as described (1). Reactions were carried out at room temperature in the following basic medium: 0.2 M sucrose, 1 mg/ml BSA, 20 mM Tes, and 20 mM KCl, adjusted to pH 7.5 with KOH.

1 This research was supported by the Department of Energy Grant EY 76-S-02-0790.
2 Abbreviations: C-ATR: carboxyatractyloside; AdN: adenosine nucleotide.
but without phosphate, and determining if addition of $[^3]H$ADP ± Pi produced $^{32}$Pi efflux and $[^3]H$ADP influx. Figure 2A shows that unlabeled Pi will exchange for endogenous $^{32}$Pi, judging by the loss of $^{32}$Pi, but that ADP will not. When the uptake of $[^3]H$ADP is followed (Fig. 2B), there is some $[^3]H$ADP uptake in absence of added Pi but without any loss of endogenous $^{32}$Pi content. If Pi is added with $[^3]H$ADP there is loss of endogenous $^{32}$Pi and additional $[^3]H$ADP uptake takes place. However, this loss of endogenous $^{32}$Pi is independent of the presence of ADP (Fig. 2A), and it must be concluded that it is the exogenous Pi in Figure 2B which has promoted $[^3]H$ADP influx. It should be emphasized that except for the preloading with $^{32}$Pi, these mitochondria were treated exactly like those for ADP uptake experiments (Mg$^{2+}$, oligomycin, and NADH were present) and under these conditions we find $^{32}$Pi leaving the matrix in exchange for exogenous Pi, but not in exchange for $[^3]H$ADP.

Corn mitochondria isolated in phosphate buffer have 3- to 4-fold more endogenous phosphate than mitochondria isolated in Tes buffer (8, 9). However, high endogenous phosphate mitochondria did not show energy-linked $[^3]H$ADP uptake until phosphate was added and were not superior to low phosphate mitochondria (data not shown). In previous experiments (1) mitochondria which were preincubated and loaded with phosphate, followed by treatment with mersalyl to block further phosphate transport, showed

![Graph](https://example.com/graph1.png)

**Fig. 1.** Simultaneous uptake of $^{32}$Pi and $[^3]H$ADP by corn mitochondria. Mitochondria (5 mg protein) were added to 3.6 ml basic medium in the presence of 1.25 µg of oligomycin/mg protein and 4 mm MgCl$_2$. Addition of 5.36 mm $^{32}$Pi (0.6 mCi/mmole) ± 2 mm $[^3]H$ADP (0.431 mCi/mmole) started the reaction. NADH (5 µmol/mg of protein) was added at 1 min.

If ADP were to enter in exchange for matrix phosphate, the pool of accumulated phosphate would not be expected to have an exact stoichiometric relationship with ADP influx. This possibility was investigated by preloading the mitochondria with $^{32}$Pi, resuspending in the basic medium with Mg$^{2+}$, oligomycin, and NADH,

![Graph](https://example.com/graph2.png)

**Fig. 2.** Loss of $^{32}$Pi accumulated by corn mitochondria by exchange with added $[^3]H$ADP, unlabeled Pi, or both. A: $^{32}$Pi retained in preloaded mitochondria after 3-min incubation with 5 mm Pi, 2 mm $[^3]H$ADP, or Pi + $[^3]H$ADP. Control was incubated in basic medium + 4 mm MgCl$_2$ without additions. B: uptake of $[^3]H$ADP by $^{32}$Pi-preloaded mitochondria in absence or presence of 5 mm Pi; nmol $^{32}$Pi/mg protein refers to preloaded $^{32}$Pi remaining after 3-min incubation and $[^3]H$ADP/mg protein to ADP accumulated in the same incubation.

### Table 1. Effect of Mersalyl, N-Ethylmaleimide, and Rotenone on the Ratio of $[^3]H$ADP/32Pi Accumulated

<table>
<thead>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>26.4 ± 1.4 (4)</td>
<td>36.0 ± 5.3 (4)</td>
<td>0.77 ± 0.09 (4)</td>
</tr>
<tr>
<td>Mersalyl</td>
<td>4.2 ± 0.6 (3)</td>
<td>13.3 ± 0.8 (3)</td>
<td>0.31 ± 0.03 (3)</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>17.1 ± 1.9 (3)</td>
<td>26.4 ± 2.5 (3)</td>
<td>0.65 ± 0.04 (3)</td>
</tr>
<tr>
<td>Rotenone</td>
<td>32.1 ± 1.5 (4)</td>
<td>130.7 ± 18.2 (4)</td>
<td>0.25 ± 0.06 (4)</td>
</tr>
</tbody>
</table>

Experimental conditions were as in Figure 1 with 0.5 mg of mitochondrial protein added to 0.4 ml of the basic medium containing oligomycin and MgCl$_2$. When present, 92 µM N-ethylmaleimide or 50 µM rotenone was preincubated 1 min with mitochondria before the addition of $[^3]H$ADP + $^{32}$Pi. Incubation was for 8 min. ( ) = No. of experiments ± SE.
strong inhibition of subsequent ADP transport. Although this was interpreted as being due to inhibition of phosphate exit, mersalyl would also block further phosphate entry. Thus, all evidence is consistent in indicating that energy-linked ADP accumulation requires exogenous phosphate and a functioning phosphate transporter. The influx of phosphate and ADP is not linked in any discernible stoichiometry.

**Anions Other than Phosphate.** A limited survey was made for other anions which would catalyze ADP accumulation. Other experiments had shown that addition of substrate anions would not promote ADP uptake, and citrate was a competitive inhibitor (3).

Arsenate was as effective as phosphate, and sulfate was slightly promotive (Table II). Sulfate is effective in activating citrate transport (14), and appears to enter via the phosphate transporter (4). Acetate, which is rapidly and extensively transported into corn mitochondria (22), did not promote ADP accumulation.

**Cation Requirement.** Table III gives [3H]ADP and 32Pi uptake in experiments where various cations were substituted for Mg2+ or Mn2+. Mg2+ was more effective than Mn2+ in promoting ADP uptake; Ca2+, Zn2+, and Fe3+ were somewhat less effective. Monovalent cations were without significant effect. Again, there was no stoichiometric relationship between ADP and phosphate uptake, although the di- and trivalent cations increased phosphate uptake as well as ADP uptake. Divalent cations, particularly Ca2+ and Sr2+, are known to activate phosphate uptake by plant mitochondria (10, 16, 21), producing insoluble phosphate deposits (16, 17).

Inasmuch as Mg2+ is probably the physiologically important cation, not producing phosphate deposits and lowering the chemical activity of phosphate, a study was made of Mg2+ accumulation. Under conditions giving ADP and phosphate accumulation, Mg2+ was also accumulated (Table IV). The endogenous Mg2+ content is high, and would surely satisfy any endogenous requirement for the cation. Hence, the action of Mg2+ in promoting phosphate and ADP uptake (Table III) must lie with some role at the outer surface of the inner membrane. Werbli et al. (20) have described a role for external Mg2+ in reducing the permeability of heart mitochondria to monovalent cations, and in corn mitochondria there is also evidence for reduced permeability in the presence of Mg2+ (14). This property of Mg2+ does not clarify its role in promoting phosphate and ADP transport. The fact that Mg2+ is also transported may be of significance here, but present data do not permit this as a conclusion. The Mg2+/ADP uptake ratio, based on analysis for ADN (3) and from Table IV (performed in the same conditions), approximates 0.7 for the net ADP uptake.

Carafoli et al. (6) reported for liver mitochondria that the uptake of ADP (or ATP) was quite sensitive to atracylsyle at 0.3 mM Ca2+, but became relatively insensitive as the Ca2+ concentration was raised to 3 mM. Since we find that ADP uptake is insensitive to carboxyatractysode, which is more effective than atracylsode with plant mitochondria (19), we checked ADP uptake using Ca2+ as the activating cation. Figure 3 shows that C-ATR is ineffective as an inhibitor over the range of 0.3 mM to 4.0 mM Ca2+. The response to Ca2+ appears to be biphasic compared with Mg2+, which is not biphasic (1).

An important finding with respect to Ca2+-activated ADP uptake is shown in Table V; there is no need to block ATP formation with oligomycin and/or C-ATR to secure extensive ADP accumulation, as is the case with 4 mM Mg2+ (1). However, just as with Mg2+, the Ca2+-activated ADP uptake requires phosphate. Apparently, ATP formation is being blocked by the presence of 4 mM Ca2+. Our laboratory has reported that mM concentrations of Ca2+ inhibit ATP formation in corn mitochondria by diverting respiratory energy into calcium phosphate uptake (11). The Ca2+/Mg2+ ratio determines the extent to which phosphate is diverted from ATP formation (11). Thus, Ca2+ alone produces the same response as Mg2+ plus oligomycin: diversion of energy from ATP formation into ion transport.

**DISCUSSION**

It is clear that the C-ATR-insensitive accumulation of ADP involves the simultaneous uptake of ADP, phosphate, and Mg2+. We can find no evidence to support our initial suggestion (1) that ADP might enter in exchange for phosphate; the required phosphate must be in the medium and be transported inward with the ADP. If phosphate transport is blocked, so is ADP transport. Similarly, Mg2+ must be in the medium for maximum transport, but since there is no specific inhibitor for Mg2+ transport it is not possible to demonstrate that the Mg2+ transport is required. Mg2+ promotes phosphate uptake (1), and it may be that Mg2+ promotion of ADP uptake lies with phosphate transport.

But if this is the case, why is there no consistent stoichiometry between phosphate and ADP uptake? A partial answer is that phosphate uptake is quite independent of ADP uptake (Fig. 1), and thus can be varied independently of ADP transport. Note, e.g. the large increase in 32Pi uptake induced by rotenone with only a small increase in [3H]ADP uptake (Table I). (We are still investigating the rotenone effect; it is not yet certain that it is due to site I inhibition.) Perhaps only a portion of the phosphate is

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**Table II. Effect of Different Anions on [3H]ADP Uptake**

Conditions were as in Table I with an anion substitution for phosphate as indicated. All anions used as 5 mM K-salts. Per cent uptake is calculated at 8 min after addition of 2 mM [3H]ADP + 5 mM unlabeled anion.

<table>
<thead>
<tr>
<th>Anion Added</th>
<th>% [3H]ADP Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate (control)</td>
<td>100</td>
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<tr>
<td>Arsenate</td>
<td>95</td>
</tr>
<tr>
<td>Sulfate</td>
<td>40</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>28</td>
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<tr>
<td>Acetate</td>
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<td>Pyrophosphate</td>
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<tr>
<td>None</td>
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**Table III. Effect of Different Cations on [3H]ADP and 32Pi Uptake**

Experimental conditions were as in Table I with cation substitution for magnesium as indicated. All cations added as 2 mM Cl-salts. Per cent uptake is calculated at 8 min after addition of 2 mM [3H]ADP + 5 mM Pi or ADP + 32Pi.

<table>
<thead>
<tr>
<th>Cation Added</th>
<th>% [3H]ADP Uptake</th>
<th>% 32Pi Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium (control)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Calcium</td>
<td>75</td>
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<td>Manganese</td>
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<tr>
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<td>25</td>
<td>27</td>
</tr>
</tbody>
</table>

**Table IV. Mg2+ Uptake by Corn Mitochondria under Conditions of ADP Accumulation**

Incubation conditions as in Figure 1 with NADH, MgCl2, oligomycin, and unlabeled Pi. Reaction terminated at indicated time by rapid centrifugation through silicone oil (see under "Materials and Methods"). ( ) = No. of assays in five experiments ± SE.

<table>
<thead>
<tr>
<th>Incubation (min)</th>
<th>0</th>
<th>2</th>
<th>5</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table IV. Mg2+ uptake</td>
<td>[342 ± 13]</td>
<td>3.2 ± 1.8</td>
<td>12.1 ± 2.3</td>
<td>219 ± 3.6</td>
</tr>
</tbody>
</table>

(1) Endogenous Mg2+
required for ADP + Pi + Mg\(^{2+}\) uptake, obscuring the stoichiometry. The Mg\(^{2+}\)/ADP ratio is not critically determined, but the available evidence suggests that Mg:ADP may be the transported species.

Arsenate will substitute fully for phosphate (Table II), which is to be expected on the basis of its ready transport via the phosphate transporter (5). Sulfate shows a little activity, and again this might be expected since sulfate in the absence of phosphate enters on the phosphate transporter, but with low efficiency (4). Mg\(^{2+}\) can be effectively substituted by Mn\(^{2+}\), a not uncommon finding in biochemical reactions involving phosphate transfer. As a generalization, the ADP accumulation can be secured with an anion which will enter on the phosphate transporter plus a divalent cation with the relative efficiency governed by ion species. It is significant that citrate is a competitive inhibitor of the ADP transport (3), which indicates that the third component of the transport system may be generalized as a trivalent anion of suitable molecular configuration.

What manner of transport is this? It certainly does not conform with the widely accepted substrate exchange transport mechanisms introduced by Chappell (7) and generally accepted for plant mitochondria (12, 23). It cannot be dismissed as irrelevant because it will function under state 3 conditions to increase the ADP content of the mitochondria (3) and because no other mechanism has been described for net ADP transport. The initial work of Carafoli et al. (6) suggests to us that this transport mechanism may not be confined to plant mitochondria (see particularly the control experiments using Mg\(^{2+}\) and phosphate with succinate as substrate). On the basis of inhibitor studies one can assume that the phosphate transporter is involved but that the ADN and tricarboxylate transporters are not, unless we are seeing undescribed properties of these carriers. One possibility is that under conditions of high proton-motive force, and with Mg\(^{2+}\) bound to the anions (and probably shielding negative surface charge on the membrane), there is a concerted proton-driven symport of Pi and ADP with bound Mg\(^{2+}\). The most likely candidate for the carrier is the phosphate transporter, which should be studied more exhaustively with respect to its anion transport specificity (13). There is also the alternative possibility that a special trivalent anion carrier exists which acts cooperatively with the phosphate transporter.

LITERATURE CITED