Efflux of Adenine Nucleotides from Rat Liver Mitochondria*

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SUMMARY

A rapid and selective efflux of 75 to 80% of the intramitochondrial adenine nucleotides (AdN) has been obtained by incubation with 5 mM Mg++ and 10 mM inorganic phosphate in the presence of 1 mM ethylenediaminetetraacetate, with little or no decrease in the P:O ratio, active respiratory state, or size of the pyridine nucleotide pool. The initial rate of AdN efflux is 5 nmoles per mg of protein per min at 30°. The efflux is a linear function of the [Pi] but with increasing [Mg++] exhibits an apparent Km for Mg++ of 0.6 mM with mitochondria incubated in the presence of 0.1 mM EDTA.

The rate of efflux of AdN is considerably reduced by respiratory inhibitors and uncouplers, including rotenone-malonate, dinitrophenol, arsenate, and oligomycin, or by an activator of ion transport such as valinomycin. All of these agents cause an increase in the proportion of AMP or ADP, or both, within the mitochondrion. Pi competitively reverses the inhibition of AdN efflux by arsenate and concomitantly increases the proportion of mitochondrial ATP.

The Mg++-Pi-induced efflux of AdN can be prevented by adding sufficient exogenous ADP to maintain an intra- to extramitochondrial AdN ratio of about 20. The omission of Mg++ or replacement by 1 mM Ca++ has little effect on the rate of AdN efflux, but lowers markedly the intra- to extramitochondrial AdN equilibrium ratio to 3.1 and 1.6, respectively. Addition of atractyloside to Mg++- and Pi-treated mitochondria completely reverses the inhibitory effect of exogenous ADP on the efflux of AdN, but is without effect on the Ca++-treated mitochondria.

It is concluded that the efflux of AdN from rat liver mitochondria under atractyloside sensitive conditions is associated with the AdN translocation site and is selective for ATP, while AMP and probably ADP are inert. The mechanism of action of Pi, Mg++, and EDTA on AdN efflux is presented in terms of their effect on ion transport and mitochondrial integrity.

Evidence has accumulated that a net flux of mitochondrial adenine nucleotides and pyridine nucleotides occurs in the presence of such ions as Mg++, Ca++, and inorganic phosphate (1-7). The addition of inorganic phosphate to aerobically respiring mitochondria is known to cause a loss of 260 mp absorbing compounds identified as AdN and pyridine nucleotides (1, 2), accompanied by a large amplitude membrane swelling (3) and loss of respiratory control (1, 4). Ernster (4) has postulated that the loss of mitochondrial ATP is responsible for the decrease of phosphorylative ability and loss of pyridine nucleotides. In the presence of exogenous ADP or ATP, a net uptake of AdN coupled to the energy-dependent accumulation of low levels of Ca++ (5) and Mg++-Pi (6) has also been observed.

It is now generally recognized that exogenous AdN can exchange across the inner mitochondrial membrane with endogenous AdN on a 1:1 basis by way of an adenine nucleotide "translocase" enzyme (8, 9). The specificity of this enzyme for exogenous ADP, even in the presence of excess ATP, appears to be responsible for the maintenance of a high extramitochondrial phosphorylation potential (8-10). The exchange, or translocation, of exogenous ADP and ATP can be specifically inhibited by atractyloside, without affecting endogenous AdN phosphorylation (8, 11, 12).

The purpose of this paper is to determine the conditions governing the selective efflux of endogenous AdN and the association of the efflux site with the proposed site of the AdN translocation enzyme. Such a study has been made practical with the development and refinement of techniques to separate mitochondria from the extramitochondrial medium rapidly (13, 14), allowing the accurate measurement of intramitochondrial metabolites at a certain instant of time and in a definite functional state. It is shown that an efflux of intramitochondrial AdN by way of the translocase site may be induced without a corresponding loss of either pyridine nucleotides or phosphorylative capacity.

* This investigation was supported in part by United States Public Health Service Research Grant CA-22, 936-03-4 from the National Cancer Institute, and by a grant from the Deutsches Forschungsgemeinschaft.

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† The abbreviation used is: AdN, total adenine nucleotides.
Rat liver mitochondria were isolated as previously described (15) in a medium containing 0.35 M sucrose, 20 mM triethanolamine buffer (pH 7.2), and 1 mM EDTA. Unless otherwise noted, mitochondria were separated from the incubation medium by a rapid centrifugal (layer) filtration method (13, 16). Generally, 0.5 to 0.7 ml of a mitochondrial suspension containing 3 to 5 mg of protein was centrifuged at 0°C through a 0.5-ml layer of silicone oil (Ar 100:150 (2:1), Wacker-Chemie, GmbH, Munich, Germany), into 0.2 ml of perchloric acid (specific gravity 1.095). When extramitochondrial ADP were included in the incubation medium a 0.5-ml wash layer of 0.25 M sucrose and 15 mg of dextran per ml was interposed between the silicone and mitochondrial layers. After centrifugation, the mitochondrial pellet and the acid-soluble supernatant fraction were frozen in a petroleum-ether-Dry Ice bath and the silicone layer was removed by solubilizing with petroleum-ether. The acid-soluble and-insoluble mitochondrial fraction was subsequently rehomogenized to assure complete extraction of ADN.

The content of intramitochondrial adenine nucleotides was measured either enzymatically (17) or as the loss of radioactivity of mitochondria prelabeled with adenine nucleotides. Preliminary experiments revealed that identical values could be obtained by both methods. When the loss of radioactivity was used, the endogenous mitochondrial nucleotides were first completely exchanged with exogenous 3H-ADP or 3H-ATP for 15 to 30 min at 0°C and twice washed in sucrose-EDTA-triethanolamine buffer. For the determination of total pyridine nucleotides, mitochondrial suspensions containing about 5 mg of protein in 0.2 ml of

### Table I

<table>
<thead>
<tr>
<th>Condition</th>
<th>Efflux, ADN</th>
<th>Respiratory control</th>
<th>ADP-O</th>
<th>Active respiration (25°C)</th>
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<tr>
<td>0 mM Mg^2+</td>
<td>0</td>
<td>3.2</td>
<td>1.8</td>
<td>316</td>
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<td>2.8</td>
<td>1.8</td>
<td>117.0</td>
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<tr>
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<td>1.9</td>
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<td>117.0</td>
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<td>81.6</td>
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<tr>
<td>0 mM additions</td>
<td>12.9</td>
<td>2.9</td>
<td>1.6</td>
<td>62.8</td>
</tr>
</tbody>
</table>

**Methods**

Rat liver mitochondria were isolated as previously described (15) in a medium containing 0.35 M sucrose, 20 mM triethanolamine buffer (pH 7.2), and 1 mM EDTA. Unless otherwise noted, mitochondria were separated from the incubation medium by a rapid centrifugal (layer) filtration method (13, 16). Generally, 0.5 to 0.7 ml of a mitochondrial suspension containing 3 to 5 mg of protein was centrifuged at 0°C through a 0.5-ml layer of silicone oil (Ar 100:150 (2:1), Wacker-Chemie, GmbH, Munich, Germany), into 0.2 ml of perchloric acid (specific gravity 1.095). When extramitochondrial ADN were included in the incubation medium a 0.5-ml wash layer of 0.25 M sucrose and 15 mg of dextran per ml was interposed between the silicone and mitochondrial layers. After centrifugation, the mitochondrial pellet and the acid-soluble supernatant fraction were frozen in a petroleum-ether-Dry Ice bath and the silicone layer was removed by solubilizing with petroleum-ether. The acid-soluble and-insoluble mitochondrial fraction was subsequently rehomogenized to assure complete extraction of ADN.

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### Table I

**Relationship between efflux of adenine nucleotides and respiratory state**

Mitochondria (5.7 mg of protein per ml), in a total volume of 1.0 ml, were shaken for 10 min at 30°C in a medium consisting of 0.25 M sucrose, 20 mM triethanolamine buffer (pH 7.2), and 1 mM EDTA, plus ions as listed. After incubation, the suspension was rapidly cooled to 0°C and centrifuged for 30 sec in a microcentrifuge, and the efflux of ADN was determined by measuring the 14C-ADP in the supernatant. Respiration was subsequently measured with a Clark oxygen electrode, with succinate as substrate. The rates of translocation of exogenous nucleotides, as well as the phosphorylation of ADP, were measured in mitochondria. Enzymes of ADP were incubated with 5 µM m-chlorobenzoyl-phenylhydrazide phenylhydrazide for 5 min, 30°C, with constant shaking to achieve full oxidation of uridine nucleotides, followed by extraction with perchloric acid. Oxidized TPN and DPN were assayed enzymatically (15, 17), at 366 nm, with the use of cuvettes with 2-cm light paths. Inorganic phosphate was measured on the acid-soluble extracts by a modification of the method of Lindberg and Ernster (18), with SnCl2 as the reducing agent, and recording the absorbance at 730 nm with a Zeiss PMQ II spectrophotometer. Mg++ was determined by atomic absorption spectrophotometry, with 3200 ppm of La(NO3)3 in 0.1 N HCl to eliminate interference by phosphate. In both methods, corrections were made for adherent extramitochondrial P1 or Mg++ by 14C-carboxypoly-glucone (16).

Radioactivity was determined with a Packard liquid scintillation counter. For counting 3H-AdN, an acidified mitochondrial extract was pipetted directly into toluene containing 20% ethanol, 4 g per liter of 2,5-diphenyloxazole, 0.1 g per liter of 2-p-phenylenedis 5-phenyloxazole, and 1% Hyamine (Packard Instrument Company). For 14C-AdN, an aliquot of a neutralized extract was pipetted onto glass fiber discs, dried, and counted in a toluene-1,4-bis(2-5-phenyloxazolyl)benzene-2,5-diphenyloxazole mixture.

Protein was determined by a biuret method (19), with KCN to eliminate interference by phospholipids. Unless otherwise noted, the cations and anions were added to mitochondria as the K+ salt.

### RESULTS

**Conditions for Depletion: Effects on Respiration, Phosphorylation, and ADN Translocation**—In Table I, conditions are presented under which a rapid efflux of ADN is obtained, together with the maintenance of a high degree of coupled respiration. In the complete efflux medium consisting of 5 mM MgCl2, 10 mM inorganic phosphate (pH 7.4), and 10 mM Tris-SO4, 75% of the endogenous ADN are lost by 10-min treatment at 30°C, compared to 13% in the control mitochondria suspended in a sucrose-EDTA-triethanolamine buffer solution. The respiratory control, ADP-O ratio, and active respiration are unaffected in the depleted mitochondria, showing that only a small fraction—less than 25%—of the complement of mitochondrial ADN is necessary for full phosphorylation capacity. It is recognized that there may be a further efflux of ADN during the subsequent measurement of controlled respiration, but this should make the amount necessary for a maximal P:O ratio less than 25%. Although not shown in Table I, measurements of the intramitochondrial ADN content during State III respiration showed that the addition of ADP to initiate active respiration had no effect on the total intramitochondrial ADN level. Omission of either the inorganic phosphate anion, or to a lesser extent, the sulfate anion, reduces the efflux of adenine nucleotides. The enhancement of efflux by sulfate is considerably less than that caused by Pi, and in most experiments was omitted, phosphate being used as the sole permeant anion. Lack of either the divalent cation Mg++ or of both phosphate and sulfate completely prevented efflux of adenine nucleotides.

The rates of translocation of exogenous nucleotides, as well as the phosphorylation of ADP, were measured in mitochondria. 

2 Other experiments have revealed that MgSO4 is as effective in promoting efflux as Tris-SO4, but MgCl2 alone is ineffective.
FIG. 1. Effect of Mg++ and phosphate on the release of adenine nucleotides. In the preparation of the mitochondria, EDTA was omitted from the sucrose-triethanolamine buffer during the final two washes. Mitochondria (4.8 mg of protein per ml) were incubated at 30°C for 4 min with sucrose-triethanolamine buffer plus 0.1 mM EDTA, keeping either the Mg++ constant at 5 mM and varying the phosphate, or keeping the P_1 constant at 5 mM and varying Mg++. Loss of AdN was measured radioactively after exchanging with ¹⁴C-ADP.

depleted of most of their stores of endogenous adenine nucleotides. No change in the rate of ADP translocation was found in mitochondria depleted of 61% of endogenous AdN. Similarly, when the rate of exogenous ADP phosphorylation was examined, a value of 29 to 30 nmoles per mg of protein per min, 10⁶, was observed for both normal and depleted mitochondria. Although accurate determination of the rate of endogenous ADP phosphorylation was limited by the low amounts of ADP present in depleted mitochondria, no observable differences were detected. The conditions governing the efflux of AdN therefore have no measurable effect on the ability of these mitochondria to translocate and phosphorylate exogenous ADP.

Concentration of P_1 and Mg++ Required for Efflux of AdN—Although both the divalent cation Mg++ and an anion must be present to achieve efflux of adenine nucleotides, the requirements for both are dissimilar, as Fig. 1 shows. In the presence of 5 mM MgCl₂, there is a monotonic increase in AdN efflux with increasing phosphate concentrations, suggestive of a competition between the phosphate and AdN anions. When inorganic phosphate is held constant at 5 mM, and MgCl₂ is varied, there is a marked activation of the efflux at low concentrations of Mg++, followed by an asymptotic approach to a maximum efflux rate. The apparent K_m by Mg++ is about 0.6 mM in the presence of 0.1 mM EDTA.

The marked dependence of the AdN efflux upon the EDTA concentration is described in Fig. 2. After labeling with ¹⁴C-ADP, the mitochondria were washed twice in an EDTA-free sucrose medium containing 20 mM triethanolamine buffer, pH 7.4. Fig. 2 shows that such mitochondria, incubated for 4 min at 30°C with 10 mM inorganic phosphate, lost 57% of their endogenous AdN, nearly as much as the 62% observed on addition of 5 mM MgCl₂. As the EDTA concentration was increased, there ensued a rapid inhibition of AdN efflux, an apparent K by Mg++ about 0.6 mM in the presence of 0.1 mM EDTA.

Relationship of P_1 and Mg++ Uptake to AdN Release—The proportionality between P_1 concentration and AdN efflux suggested that the efflux may be a simple counterexchange of the AdN and P_1 anions. However, when mitochondrial inorganic phosphate was measured under identical conditions as in Fig. 2, no positive relationship was found to exist between the degree of efflux of AdN and uptake of inorganic phosphate (Table II). In fact, there was a slight increase in the amount of phosphate incorporated as the efflux of AdN was retarded by increasing amounts of EDTA. Thus, in accordance with its known properties as a permeant anion (20), phosphate can be rapidly taken up under these conditions, but is incapable of causing the release of AdN until the EDTA concentration has been sufficiently reduced. These observations are consistent with the postulate (19, 21) that EDTA renders the mito-

![Graph](http://www.jbc.org/)

TABLE II

<table>
<thead>
<tr>
<th>Additions</th>
<th>Inorganic phosphate</th>
<th>AdN</th>
</tr>
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<tbody>
<tr>
<td>No incubation</td>
<td>10.6</td>
<td>14.7</td>
</tr>
<tr>
<td>None</td>
<td>28.9</td>
<td>8.1</td>
</tr>
<tr>
<td>5 mM Mg++</td>
<td>31.4</td>
<td>7.2</td>
</tr>
<tr>
<td>10 μM EDTA</td>
<td>26.7</td>
<td>8.7</td>
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<tr>
<td>40 μM EDTA</td>
<td>35.2</td>
<td>11.5</td>
</tr>
<tr>
<td>100 μM EDTA</td>
<td>40.9</td>
<td>13.1</td>
</tr>
</tbody>
</table>

3 H. Heldt and H. Meisner, unpublished data.
MINUTES
FIG. 3. A comparison of the release of AdN and pyridine nucleotides (PN) from mitochondria. Duplicate samples containing 5.5 mg of protein per ml were incubated at 30° in sucrose-triethanolamine-EDTA plus 5 mM Mg++, 10 mM Pi, and 10 mM Tris-SO₄. AdN and pyridine nucleotides were measured enzymatically as described under "Methods," after separation by centrifugation.

FIG. 4. Rate of efflux of adenine nucleotides from rat liver mitochondria. Mitochondria (4.7 mg of protein per ml) were incubated at 30° in sucrose-triethanolamine-EDTA medium plus 5 mM Mg++, 10 mM Pi, and 10 mM Tris-SO₄, and separated by centrifugal filtration at the indicated times. AdN was measured enzymatically, with the initial AdN content of the mitochondria being 12.9 nmoles per mg of protein.

The mitochondrion more permeable to alkali metal cations and protons. In this manner, entering Pi anions are neutralized by accompanying cations, and the counterexchange with AdN is prevented.

When EDTA is omitted from the Pi incubation medium, Table II indicates that Mg++ is not required for the efflux of AdN. The efflux in the absence of EDTA and Mg++, however, is known to be accompanied by a loss of pyridine nucleotides (1, 2) and respiratory control (1, 4).

Measurement of the Mg++ content of mitochondria after efflux of AdN in the presence and absence of external Mg++ revealed no change in the intramitochondrial Mg++ level of 25 to 28 nmoles per mg.

Selectivity of Efflux for Adenine Nucleotides—It is known (2, 3, 22) that a severe depletion of both pyridine nucleotides and AdN can be observed by incubating mitochondria with 10 mM Pi. On the other hand, hypotonic conditions such as water or 10 to 20 mM Tris buffer cause little or no loss of pyridine nucleotides (3, 7), but lead to a loss of AdN as well as respiratory control (7). To determine the specificity of nucleotide loss in mitochondria incubated with Mg++ and Pi, the rate of AdN efflux was compared simultaneously to the loss of total pyridine nucleotides. Fig. 3 shows that the initial rate of leakage of pyridine nucleotides is about 10% that of the AdN efflux, clearly revealing the highly selective nature of the efflux for AdN under these conditions. The major part of the loss of total pyridine nucleotides occurs after the cessation of AdN efflux, and is therefore not associated with the factors governing AdN leakage.

Kinetics of Efflux—When the kinetics of efflux of AdN was examined (Fig. 4), a marked loss was observed in the first few minutes. These mitochondria lose in 6 to 7 min about 77% of their complement of AdN, and show an initial rate of efflux of 5.2 nmoles per mg of protein per min. Control mitochondria lose at the same time, and in a linear fashion, about 7% of their AdN.

Steady State Ratios of Intra-Extramitochondrial Adenine Nucleotides—One aspect of Fig. 4 is now discussed, namely, the failure to observe here, as well as in other experiments, a 100% loss of AdN. In Table III, mitochondria were incubated in a cation-anion medium to steady state intramitochondrial AdN level was attained. In Row 1, it has been estimated that after 10 min of efflux the steady state ratio of intra- to extramitochondrial AdN in depleted mitochondria is 23. If ADP is added initially (Row II), 0.4 mM exogenous ADP is sufficient to balance the influx potential for adenine nucleotides, yielding an intra- to extra-

![Graph]

TABLE III
Ratio of intra- to extramitochondrial adenine nucleotides during steady state flux conditions

Mitochondria were incubated in sucrose-triethanolamine-EDTA at 30° for 10 min in 5 mM Mg++ and 10 mM phosphate (I and II), or for 5 min with 1 mM Ca++ and 5 mM phosphate (III), or for 10 min in 10 mM Pi (IV). These times were found to be adequate for a steady state level of exogenous AdN to be reached. EDTA was omitted from the last two mitochondrial washes as well as the incubation medium in Experiment IV. Sufficient ADP was added in Experiments II, III, and IV to prevent exactly net flux of endogenous AdN. In Experiment I, the concentration of extramitochondrial AdN was estimated from the loss of intramitochondrial AdN. In Experiments II, III, and IV, extramitochondrial AdN refers to the amount of ADP added. Intramitochondrial AdN concentration was determined enzymatically, after correcting for adherent AdN by 4-C-carboxy-polyglucose, and assuming an osmotically impermeable matrix space of 2.0 μl per mg of protein (9).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Additions</th>
<th>(A) Intramitochondrial AdN</th>
<th>(B) Extramitochondrial AdN</th>
<th>A:B</th>
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<tr>
<td>I</td>
<td>Mg++ + phosphate</td>
<td>1.20</td>
<td>0.053</td>
<td>23</td>
</tr>
<tr>
<td>II</td>
<td>Mg++ + phosphate + ADP</td>
<td>7.45</td>
<td>0.40</td>
<td>19</td>
</tr>
<tr>
<td>III</td>
<td>Ca++ + phosphate + ADP</td>
<td>6.85</td>
<td>4.3</td>
<td>1.6</td>
</tr>
<tr>
<td>IV</td>
<td>Phosphate + ADP</td>
<td>7.03</td>
<td>2.25</td>
<td>3.1</td>
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</table>
mitochondrial ratio of 19. The failure to observe a total leakage of intramitochondrial AdN can therefore be explained by the build-up of exogenous AdN to a point at which the tendency to efflux is exactly balanced by the influx. This was confirmed by experiments in which greater final efflux values of AdN were obtained by simply reducing protein concentrations, thereby creating a lower extramitochondrial AdN concentration. In the third row, it is seen that, during efflux induced by 1 mM Ca++ plus 5 mM phosphate, considerably higher extramitochondrial AdN is necessary to prevent net flux, and the AdN ratio is near unity (1.6). If mitochondria prepared by washing twice without EDTA are treated with 10 mM P1 as the sole ion (Row IV), a ratio of 3.1 is required to balance AdN flux. The striking properties of the efflux reaction maintained by the Mg+f cation. Endogenous AdN during efflux is shown in Table IV. In the control experiments, there is a very slow loss of AdN but a large shift of the ATP:AMP ratio. Treatment with Mg++ and Pi yields a rapid loss of adenine nucleotides, but the proportions of intramitochondrial ATP, ADP, and AMP remain constant until 20 min, at which time there is a slight shift in the ADP-AMP pattern. As we show below, the constancy of the proportions of AdN during ion-induced efflux occurs in spite of a selective loss of one AdN species. It is probable that the selective efflux of AdN is effectively “masked” by a stable phosphate potential maintained by a high intramitochondrial phosphate concentration.

Selectivity of Efflux for ATP.—To gain information regarding the specificity of the leakage toward the form of AdN, dinitrophenol and arsenate were added. After a 1-min pretreatment of the mitochondria with dinitrophenol or arsenate, 86% of the adenine nucleotides was present as ADP and 65% as AMP respectively (Table V). In Table V, it can be seen that the initial rate of the Pi-Mg++-induced efflux is reduced 80% by pretreatment with dinitrophenol or arsenate.

### Table IV

<table>
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<th>Additions</th>
<th>Time at 30°</th>
<th>AdN as</th>
<th>Efflux AdN</th>
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<td></td>
<td>min</td>
<td>AMP</td>
<td>ADP</td>
</tr>
<tr>
<td>None</td>
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<td>26</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>34</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>66</td>
<td>34</td>
</tr>
<tr>
<td>Mg++ + phosphate + Tris-SO₄</td>
<td>2</td>
<td>16</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>15</td>
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<td>15</td>
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<td>20</td>
<td>39</td>
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### Table V

<table>
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<tr>
<th>Experiment and inhibitor</th>
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<td></td>
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<td>Arsenate</td>
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<td>40</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>6</td>
<td>23</td>
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</table>

* Corrected for efflux occurring during the 1-min incubation with dinitrophenol or arsenate.

Fig. 5. Inhibition of adenine nucleotide efflux by dinitrophenol and arsenate. Mitochondria (6.7 mg of protein per ml) were incubated in sucrose-triethanolamine-EDTA medium and pretreated with either 5 mM arsenate or 0.08 mM dinitrophenol for 1 min, 30°, followed by addition of 5 mM Mg++, 10 mM phosphate, and 10 mM Tris-SO₄. Samples were removed at the intervals indicated and separated by centrifugal filtration, and AdN was measured enzymatically.
Effect of inhibitors on efflux of adenine nucleotides

Mitochondria (4.4 mg per ml) were incubated for 4 min at 30° in sucrose-triethanolamine-EDTA medium plus 5 mM Mg++ and 10 mM phosphate. The following concentrations of inhibitors were used: KCN, 5 mM; oligomycin, 2.5 μg per ml; malonate, 5 mM; rotenone, 1 μM; dinitrophenol, 0.08 mM; and arsenate, 5 mM. The loss of ADN was measured radioactively while AMP and ADP were determined by enzyme assay.

Additions | ADN as AMP + ADP | Efflux ADN
---|---|---
No incubation | % | %
None | 49 | 80
KCN | 92 | 34
Oligomycin | 95 | 86
Malonate + rotenone | 99 | 34
Dinitrophenol | 100 | 30
Dinitrophenol + oligomycin | 92 | 36
Arsenate | 93 | 27

* Mitochondria were pretreated for 1 min, 30°, with either dinitrophenol or arsenate.

Prevention of adenine nucleotide efflux by valinomycin and K+

Mitochondria (4.2 mg of protein per ml) were isolated in a K+-free sucrose-triethanolamine buffer, and incubated for 5 min at 30° in 5 mM Mg++ and 10 mM Na+ phosphate. When added, the concentration of K+ was 5 mM and valinomycin was 0.3 μg per ml. Efflux of ADN was measured as loss of radioactivity, and ADP enzymatically.

Additions | Efflux ADN | ADN as ADP
---|---|---
None | % | %
K+ | 5 | 49
Valinomycin | 74 | 55
K+ | 75 | 60
K+ + valinomycin | 70 | 60

* Mitochondria were incubated for 5 min, 30°, without Mg++ and phosphate.

Effect of atractyloside on efflux of adenine nucleotides

Mitochondria were treated for 5 min at 30° in sucrose-triethanolamine-EDTA with 5 mM Mg++ and 10 mM phosphate, and separated by filtration layer centrifugation. Succinate, 5 mM and 1 mM ADP were added when indicated. In Experiment I, efflux of ADN was measured radioactively after exchanging with 14C-ADP. In Experiment II, ADN was measured enzymatically. Experiment I, 3.8 mg of protein per ml. Experiment II, 4.4 mg of protein per ml.

<table>
<thead>
<tr>
<th>Experiment and additions</th>
<th>Atractyloside</th>
<th>Efflux ADN</th>
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<td>μM</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>I. None</td>
<td>0</td>
<td>57</td>
</tr>
<tr>
<td>10</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>II. Succinate</td>
<td>0</td>
<td>82</td>
</tr>
<tr>
<td>Succinate + ADP</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>50</td>
<td>71</td>
<td></td>
</tr>
</tbody>
</table>

ADN as AMP + ADP to 18%, and a concomitant 2-fold increase in the efflux above that found with dinitrophenol present. The inhibitory effect of arsenate on ADN efflux could be completely eliminated by decreasing the concentration to 2 mM (Experiment II). Under these conditions, the Pt anion was able to compete more effectively with arsenate, and the percentage of ATP was increased from 8 to 71% 2 min after the addition of the efflux medium.

From the preceding data, it can be postulated that dinitrophenol and arsenate act either by decreasing the level of an "~X" intermediate necessary to drive an anion pump required for the efflux of ADN, or simply by changing the adenine nucleotide to a less easily effluxible form. It is more likely, as evidenced in Table VI, that the rate of efflux is controlled, at least in part, by the proportion of the ADN in the form of ATP. Oligomycin, added alone or with dinitrophenol, reduced the efflux of ADN from 80% to 36% in 4 min, at which time about 85% of the endogenous ADN was in the form of AMP plus ADP. Addition of cyanide and malonate plus rotenone, dinitrophenol, or arsenate also lowered the efflux to about 35%, with AMP and ADP comprising 90 to 100% of the remaining ADN. Further experiments have shown that the addition of succinate to raise the ~X level has no inhibitory effect on the efflux. It is nevertheless possible that the efflux via the ADN translocase carrier requires a high energy intermediate of oxidative phosphorylation, but the conformational state of the membrane in the presence of oligomycin is unfavorable for the loss of ADN.

The striking inhibition of the ADN efflux by valinomycin is shown in Table VII. When added separately, neither valinomycin nor 5 mM K+ had any effect on the Mg++-phosphate-induced efflux but, when added together, reduced efflux by about 90%. Furthermore, 90% of the remaining ADN in the valinomycin-K+ mitochondria was present as ADP, as opposed to between 50 and 60% under the other conditions. The large accumulation of ADP in the K+-valinomycin treated mitochondria indicate that, in this case, the active transport of K+ successfully competed for the ATP produced through oxidative phosphorylation (23). These data, as well as the preceding, support the postulate that ATP is the form of ADN primarily effluxed from the mitochondrion, and that AMP and ADP are relatively inactive under these conditions of leakage.

Effect of Atractyloside on Efflux—Atractyloside, known to inhibit the phosphorylation of exogenous ADP and the exchange reaction (9, 11) by preventing the binding of ADP and ATP to the translocase site (24), was used to define better the location of the ADN efflux. It is seen in Table VIII that up to 500 μM atractyloside had no inhibitory effect on the ADN efflux. Addition of 1 mM external ADP completely blocked the efflux and, in fact, led to a slight increase in the level of endogenous ADN. The further addition of atractyloside almost completely prevented the inhibitory effect of ADP. When succinate was added in addition to Mg++ and phosphate, a slight stimulation of the efflux was noted. The stimulatory effect of substrate has also been found in other experiments, and is attributed to its ability to act as a permeant anion, in a similar manner to Pi uptake revealed that oligomycin-treated mitochondria accumulate as much Pi as controls. Thus the energy necessary to drive the uptake of Pi is available, but ADN efflux is limited by other factors.

Concentrations of atractyloside between 0.5 and 1.0 n mole per mg of protein have been found to stimulate the efflux of ADN after a lag period of 1 to 2 min (H. Meisner, unpublished data).
ADP are necessary to prevent net AdN efflux, and (b) parallel nucleotides. It has been reported that the uptake of adenine produced by 1 mM ADP prevented net AdN efflux, and cause AdN efflux (see Table I), the data in Table IX emphasize increased, considerably higher levels of extramitochondrial indicating that (a) as the external concentration of Ca++ is membrane. These results lend support to the data of Table III, the destructive nature of 1 mM Ca++ on the inner mitochondrial wlesh. Incubation was carried out for 5 min (Experiment I) or 4 min (Experiment II) at 30°, followed by separation of the mitochondria from the medium by filtration layer centrifugation at 0°. Intramitochondrial adenine nucleotides were measured enzymatically. Experiment I: 6.1 mg of protein per ml; Experiment II: 4.8 mg of protein per ml.

**Table IX**

*Properties of Ca++-induced efflux of adenine nucleotides: effect of atractyloside*

In the preparation of the mitochondria, a 0.1 mM EDTA-sucrose-triethanolamine medium was used during the final two washes. Incubation was carried out for 5 min (Experiment I) or 4 min (Experiment II) at 30°, followed by separation of the mitochondria from the medium by filtration layer centrifugation at 0°. Intramitochondrial adenine nucleotides were measured enzymatically. Experiment I: 6.1 mg of protein per ml; Experiment II: 4.8 mg of protein per ml.

<table>
<thead>
<tr>
<th>Experiment and efflux medium</th>
<th>Additions</th>
<th>Efflux AdN percentage of total mitochondrial AdN</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>6.3 mM Ca++ + 5 mM phosphate</td>
<td>0 0 77</td>
</tr>
<tr>
<td></td>
<td>0.5 0.05 75</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>1 mM Ca++ + 5 mM phosphate</td>
<td>1 0.05 61</td>
</tr>
<tr>
<td></td>
<td>1 0 60</td>
<td></td>
</tr>
</tbody>
</table>

**Table X**

*Properties of Ca++-induced efflux of adenine nucleotides: effect of energy inhibitors*

Mitochondria (5.9 mg of protein per ml) were incubated for 4 min at 30° in sucrose-triethanolamine-0.1 mM EDTA plus 1 mM Ca++ and 5 mM phosphate, separated by filtration centrifugation at 0°, and AdN was determined enzymatically. When indicated, the following inhibitors were added: dinitrophenol, 0.08 mM; arsenate, 5 mM; valinomycin, 0.1 μg per ml; and K+, 5 mM.

<table>
<thead>
<tr>
<th>Additions</th>
<th>AdN as AMP + ADP</th>
<th>Efflux AdN percentage of total mitochondrial AdN</th>
</tr>
</thead>
<tbody>
<tr>
<td>No incubation</td>
<td>65</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>89</td>
<td>82</td>
</tr>
<tr>
<td>Dinitrophenol</td>
<td>93</td>
<td>67</td>
</tr>
<tr>
<td>Arsenate</td>
<td>84</td>
<td>84</td>
</tr>
<tr>
<td>Valinomycin + K+</td>
<td>97</td>
<td>69</td>
</tr>
</tbody>
</table>

phosphate. Again, 1 mM ADP prevented net AdN efflux, and atractyloside reversed this blocking effect of ADP.

**Effect of Atractyloside on Ca++-induced Efflux of Adenine Nucleotides**—It has been reported that the uptake of adenine nucleotides in the presence of low levels of Ca++ occurs via an atractyloside-sensitive pathway (5). Table IX shows that 0.3 mM Ca++ can also induce an atractyloside-specific efflux of AdN and that this specificity is lost when Ca++ is increased to 1 mM. There is a 57% loss of AdN when 1 mM Ca++ is added in the absence of Pi. Compared to the inability of Mg++ to cause AdN efflux (see Table I), the data in Table IX emphasize the destructive nature of 1 mM Ca++ on the inner mitochondrial membrane. These results lend support to the data of Table III, indicating that (a) as the external concentration of Ca++ is increased, considerably higher levels of extramitochondrial ADP are necessary to prevent net AdN efflux, and (b) parallel with this, the inhibitory effect of atractyloside disappears.

More evidence for the unspecificity of the efflux of AdN induced by 1 mM Ca++ is found in Table X. There is little or no inhibitory effect of dinitrophenol, arsenate, or valinomycin on the efflux of AdN, yet there is a marked increase in the proportion of the remaining intramitochondrial AMP and ADP. It will be recalled that these inhibitors greatly retarded the efflux of AdN when Mg++ was the source of the divalent cation, and caused an increase in the proportion of AMP and ADP. The failure of the increased AMP and ADP levels to retard AdN efflux is thought to be a result of the Ca++-induced loss of specificity of the efflux at the inner mitochondrial membrane for ATP or, conversely, the increased permeability of AMP and ADP.

**Discussion**

**Specific and Unspecific AdN Efflux**—It has been previously thought that a loss of AdN content and respiratory control by swelling agents were necessarily parallel phenomena (1, 4). The depletion of the mitochondrial pool of endogenous AdN by incubation with Mg++ and P, contradicts this assumption, as these mitochondria retain the capacity for the oxidation of substrates and oxidative phosphorylation. At least 75% of the pool of intramitochondrial AdN, therefore, appears not to be required for an efficient oxidative phosphorylation of added ADP by isolated mitochondria, although it is not excluded that the full pool of endogenous AdN may have certain regulatory functions.

The efforts of previous authors (1, 3) to deplete mitochondria of endogenous nucleotides with P, so the sole ion leads to an extensive loss of AdN and pyridine nucleotides, while at the same time the capacity for oxidative phosphorylation is lost and the oxidation of various DPN-linked substrates is largely impaired. The preservation of respiratory capacity under the present conditions can be related to the retention of pyridine nucleotides within the matrix space during the simultaneous loss of adenine nucleotides. Thus the depletion of nucleotides from mitochondria treated with Pi or Ca++ as the sole swelling agents can now be classified as an unspecific type of efflux, in contrast to the specific efflux, as established in the present studies, in which AdN and essentially no pyridine nucleotides are lost.

**Influence of Mg++, Phosphate, and EDTA on AdN Efflux**—The effectiveness of P, in depleting mitochondrial nucleotides has been noted in earlier reports (1, 2), and several roles of Pi may be considered, all dependent upon its properties as an easily permeable anion. The phosphate-induced release of AdN resembles an anion exchange, with Pi accumulating in excess to AdN and then competing with cations such as Mg++ and possibly cationic groups of proteins, thus initiating a counterexchange with the pool of mitochondrial nucleotides. It should be considered in this context that the rate and extent of Pi influx are some orders of magnitude higher than the AdN efflux. In this manner, the uptake of P, serves to drive the efflux in the specific as well as in the nonspecific case.

As is well documented, Pi induces a swelling of the mitochondrial matrix (3, 25), causing a conformational change of the membrane that is apparently required for the efflux of nucleotides. Diverse agents such as EDTA, dinitrophenol, or oligomycin are known to cause a contraction of reversibly swollen mitochondria (3, 21) and markedly reduce AdN efflux.

The accumulation of P, within the matrix would tend to increase the ATP:ADP ratio in mitochondria maintained at a constant phosphorylation potential, i.e. ATP:ADP:Pi (20). This particular role of Pi is effective in the specific efflux, when ATP
is the only observable effluxing species, and a high ATP content would enhance loss of AdN. The importance of the role of P_i in preserving a constant ATP:ADP ratio is underlined by the effect of arsenate. Although arsenate is able to penetrate the mitochondrion quite rapidly and induce swelling (3), its uncoupling action and consequent dephosphorylation of ATP yields a low specific efflux of AdN. Correspondingly, the inhibition of the specific AdN efflux by respiratory uncouplers or inhibitors may also be the dual result of a decrease of the ATP content and prevention of mitochondrial swelling.

An important factor in the effect of Mg++ and EDTA on AdN efflux appears to be their influence on mitochondrial swelling and membrane integrity. In the absence of Mg+++, the P_i-induced loss of pyridine nucleotides and AdN is accompanied by a high amplitude swelling (3, 25). In the presence of EDTA, the P_i swelling is largely if not completely inhibited (3, 27), and the efflux of pyridine nucleotides (22) as well as AdN (this report) is prevented. An excess of Mg++ yields a low amplitude type of swelling (25, 28) and, as reported here, affords a specific loss of AdN but a simultaneous prevention of pyridine nucleotide efflux. The maintenance of the specificity of efflux clearly represents a physiologically beneficial effect of Mg++ on the mitochondrial membrane. It therefore appears that the conformational change of the inner membrane induced by phosphate yields at a low amplitude swelling (+Mg++) a specific AdN efflux, and at large amplitude swelling (−Mg++) an unspecific efflux of pyridine nucleotides and AdN.

The mechanism of the influence of Mg++ or EDTA on the efflux is largely open to speculation. Under present conditions, little or no uptake of Mg++ into rat liver mitochondria has been observed, in agreement with reports from other laboratories (3, 0, 29). It may be considered that the function of Mg++ is to preserve the integrity of the mitochondrial membrane, in this manner allowing the efflux of AdN anions in exchange for P_i anions through a membrane in a state of controlled swelling. Addition of EDTA in the absence of Mg++ is considered to inhibit the P_i-induced swelling (3) and increase cation permeability (27), in this manner balancing the uptake of P_i with an equally rapid accumulation of protons or alkali metal cations.

Relation of AdN Efflux to Translocase Site—In a comparison of the AdN efflux with the AdN translocase, it should be recognized that the rate of translocation of external ADP (about 400 to 500 nmoles per mg per min) is nearly 100 times faster than the rate of AdN efflux (5 nmoles per mg per min) when both are measured at 30°C. Assuming that the efflux and exchange site are identical, it is likely that, in the comparatively slow efflux of AdN, either there is not a strict 1:1 exchange of the exogenous and endogenous AdN or P_i is exchanged in place of ADP and ATP for the endogenous AdN.

The following reasons favor the association of the specific efflux of mitochondrial AdN with the proposed AdN translocase (9). First, the insignificant loss of pyridine nucleotides in the specific AdN efflux is analogous to the absence of a pyridine nucleotide exchange in the presence of an extremely rapid AdN exchange (10). Another argument is the reduction of AdN efflux by concentrations of external adenine nucleotides (0.02 to 0.04 mM) that have been shown to activate the AdN exchange (10). When the efflux is made unspecific by 1 mM Ca++ or by a lack of Mg++, the exogenous AdN levels required to inhibit the efflux (2 to 4 mM) greatly exceed the K_m for the AdN exchange. Third, atracrynolide, an inhibitor of the AdN exchange (8, 11), releases the suppression of the specific AdN efflux by external adenine nucleotides, but has no effect on the Ca++-induced AdN efflux. Importantly, the inability of atracrynolide to retard AdN efflux indicates that it acts on the outside of the membrane, and does not diffuse or translocate into the mitochondrion, as has been previously implied (12). A binding of atracrynolide to the outside part of the translocase would not prevent a binding of AdN to the inside part during AdN efflux.

Finally, the specificity of the efflux for ATP and apparent inertness of AMP and ADP are in partial agreement with characteristics of the exchange. AMP is not able to traverse the inner mitochondrial membrane in the efflux of AdN and also cannot be exchanged in the forward (8) or back translocation (10) reaction. The apparent inertness of endogenous ADP in the efflux reaction is more difficult to consider, in view of the high affinity of the AdN translocase for ADP (8, 30). In the AdN exchange, ATP and ADP have been shown to be back exchanged to an extent proportional to their intramitochondrial ratio (10). Assuming that the characteristics of the efflux and translocation of AdN are similar, the possibility cannot be excluded that the inhibition of AdN efflux in the presence of dinitrophenol, valinomycin-K+, or oligomycin may be unrelated to the accompanying accumulation of ADP. Dinitrophenol and valinomycin are known to increase the influx of protons (31) and K+ (32, 33), respectively, into the matrix space. The resulting neutralization of any anion excess would interfere with the P_i-AdN counterexchange and effectively prevent the efflux of AdN anions. The inhibition of P_i-induced swelling by dinitrophenol or oligomycin (3, 21, 34) may also effectively

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Fig. 6. A scheme for the release of AdN from rat liver mitochondria. The specific efflux is induced by P_i + Mg++, while the nonspecific efflux can be induced by P_i or by 1 mM Ca++ + P_i.

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*E. Pfaff, unpublished data.*
prevent the efflux of ADP by creating an unfavorable membrane configuration.

Based upon the incomplete exchangeability of external ADP or ATP with endogenous AdN (30, 35, 36), two pools of AdN or more have been postulated, one perhaps being located “in the sealed chamber of the repeating unit” (35). By directly measuring the exchange, and recognizing that the AMP pool is non-exchangeable, Pfaff (8, 10) has presented conclusive evidence that only one compartment exists for AdN. In this paper, it has been found that the residual pool of AdN in depleted mitochondria shows a rapid phosphorylation rate, and participates in a normal exchange with exogenous AdN, supporting the concept of a single pool encompassing the released and retained AdN.

Proposed Scheme for ADN Efflux—A proposed mechanism for the pathway of the specific and nonspecific AdN efflux is presented in Fig. 6. For simplicity, only the exchange diffusion carrier for Pi is represented. When Mg++ is present in addition to Pi, the efflux of AdN is specific for ATP, and occurs by way of the AdN translocase. The influx of the Pi anion, coupled to a net cation translocation of zero. In mitochondria treated with Pi, in the absence of Mg++ and EDTA, or in the presence of 1 mm Ca++ (nonspecific efflux), there is a tendency to translocate protons or cations from the matrix to the extramitochondrial space. When coupled to the rapid influx of the Pi anion, either unspecific new efflux sites are created, or the selectivity of the pre-existing translocase sites for ATP is destroyed. The membrane becomes permeable to ADP, AMP, and pyridine nucleotides, and the function of the AdN translocase is lost.

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