Kinetics and Specificity of the Adenine Nucleotide Translocation in Rat Liver Mitochondria*

E. D. Duée AND Pierre V. Vignais

From the Laboratoire de Biochimie, Centre d’Etudes Nucléaires, et Faculté de Médecine de Grenoble, 38, Grenoble, France

SUMMARY

The conditions required for identification of the translocation of adenine nucleotides as an exchange-diffusion process in rat liver mitochondria have been determined. Among them, the presence of ethylenediaminetetraacetate in the medium and a temperature not higher than 0° are the most critical ones.

Experiments performed with a phosphonic analogue of ADP, namely adenosine 5’-methylene diphosphonate, indicate that all of the adenine nucleotides within mitochondria are exchangeable.

The rate of exchange exhibits oscillation-like fluctuations immediately after ADP has been added to mitochondria. Similar fluctuations of the amount of ~P bonds in internal adenine nucleotides suggest that the exchange rate may be dependent on the phosphorylation pattern of the mitochondrial adenine nucleotides.

The apparent $K_m$ of the nucleotide-exchange system for extramitochondrial ADP is 5.6 mM in an EDTA-supplemented medium. It rises to 17 mM when EDTA is replaced by MgCl$_2$.

The adenine nucleotide translocation exhibits a $Q_{10}$ of 10 to 12 below 10° and of 2 to 3 above 10°. By contrast the $Q_{10}$ of the phosphorylation of mitochondrial ADP is 2 to 3 within the temperature range 0–25°.

Small amounts of CDP, GDP, and UDP (after any contamination by adenine nucleotides has been eliminated) are taken up by mitochondria via an atractyloside-insensitive process. On the other hand, dADP and the phosphonic analogues of ADP and ATP are exchanged with the mitochondrial adenine nucleotides via an atractyloside-sensitive process; the latter exchanges are competitively inhibited by ADP.

The translocation of ADP, and much more significantly the translocations of dADP and adenosine 5’-methylene diphosphonate, are stimulated by the addition of inorganic phosphate in the medium. These results are discussed on the basis of a preferential exchange between the internal ATP generated within mitochondria by oxidative phosphorylation and the extramitochondrial ADP.

* This investigation was supported by research grants from the Centre National de la Recherche Scientifique (RCP 21), the Fondation de la Recherche Médicale, and the Délégation Générale à la Recherche Scientifique et Technique. This material was taken from a thesis to be submitted (E. D. D.) to the Faculté des Sciences, Université de Grenoble.

Adenine nucleotides added to mitochondria are rapidly and extensively exchanged with intramitochondrial (internal) adenine nucleotides by translocation through mitochondrial membranes (1–12). The transmembrane exchange of adenine nucleotides is competitively inhibited by atractyloside (1–7, 10), and by atracytyligenin (7, 13), a property which has been useful in evaluating the dependence of several mitochondrial reactions upon extramitochondrial (external) adenine nucleotides. External adenine nucleotides are translocated into the mitochondrial matrix, probably by exchange-diffusion, since translocation occurs even when the concentration of the external adenine nucleotides is much lower than that of the internal adenine nucleotides (3, 6, 7). Although these general features of adenine nucleotide translocation are well established, several controversies have recently arisen concerning the membrane localization of the translocase system (10, 14, 15), its kinetic properties (10, 12), and its specificity (2–7, 10, 16, 17).

In this report, we describe the conditions under which the adenine nucleotide translocation occurs by exchange-diffusion. Among them, the presence of ethylenediaminetetraacetate in the incubation medium and a temperature not higher than 0° are the most critical ones. When undertaken under these specified conditions, the kinetic study of the transmembrane exchange of adenine nucleotides shows that the pool of internal adenine nucleotides is totally exchangeable and that the initial rate of exchange is influenced by factors related either to the distribution of the internal adenine nucleotides or to the rate of ATP synthesis. Data bearing on nucleotide specificity indicate that not only ADP and ATP, but also their deoxy derivatives and their phosphonic analogues, are rapidly exchanged with the internal adenine nucleotides by an atractyloside-sensitive process. On the contrary, only minute amounts of UDP, CDP, and GDP are incorporated into mitochondria; the rate and the extent of these latter incorporations are not altered by atractyloside.

MATERIALS AND METHODS

Rat liver mitochondria were isolated according to the method of Hogeboom (18) in 0.25 M sucrose solution buffered at pH 7.4 by Tris-HCl buffer (final concentration 1 mM). Digitonin particles were prepared according to the method of Devlin and Lehninger (19).

Potassium atractylate (tractylolide) was extracted from the rhizomes of the thistle Atractylis gummifera according to the method of Angelio (20) and recrystallised several times from
calculation of the sedimentation time is given in detail elsewhere. The bottom of the tube was usually stopped by the addition of atractyloside at a final concentration of 10^{-4} M, immediately followed by centrifugation at 25,000 \times g for 5 min in a SS-1 Servall centrifuge with the maximum centrifugal force being reached within 30 sec. Under these centrifugation conditions, mitochondria were collected at the bottom of the tube within a mean time of about 45 sec. The calculation of the sedimentation time is given in detail elsewhere (28).

As will be shown under “Results” (Fig. 2), 10^{-4} M atractyloside inhibits 75 to 80% of the exchange and higher concentrations of atractyloside do not substantially increase the inhibition. In order to take into account the residual incorporation, control tubes were run for each experimental condition. In these control tubes, atractyloside was added prior to mitochondria and the centrifugation was started immediately after the addition of mitochondria. This method, here called the atractyloside-centrifugation method, allows incubation periods as short as 5 sec to be attained conveniently while retaining good accuracy. After the first centrifugation in the presence of atractyloside, the mitochondrial pellet was washed three times in KCl-Tris-EDTA. The experiments reported in Table II (“Results”) show that the intramitochondrial adenine nucleotides are not washed out by this procedure. The incorporated (^{14}C)-adenine nucleotides were extracted by perchloric acid and their amount in the acid extract was estimated by liquid scintillation counting; the scintillation fluid used contained 100 g of naphthalene, 6 g of 2,5-diphenyloxazole and 300 mg of p-bis[2-(5-phenyl-oxazolyl)]benzene per liter of 1,4-dioxane.

In preliminary experiments, we compared the atractyloside-centrifugation method to other methods used to stop the translocation reaction: the standard centrifugation method, Millipore filtration, and centrifugal layer filtration. A brief account of our results follows. In the standard centrifugation method (1–4), the incubation was stopped by centrifugation at 25,000 \times g for 10 min without prior addition of atractyloside. The mitochondrial pellet was then washed and extracted as in the atractyloside-centrifugation method. However, since incubation continues during the first centrifugation, the incubation period cannot be shorter than the mean time of the pellet formation (45 sec, see above). Therefore, this method is inadequate for the determination of the rate of exchange over short periods of incubation.

Winkler, Bygrave, and Lehninger (10) recently proposed a modification of the Millipore filtration technique of Brierley and O’Brien (4). In this modification, mitochondria were harvested from the incubation medium by filtration through a Millipore filter and washed with isotonic KCl. The radioactivity of the filter was then counted. Although this method has the advantage of simplicity, its application suffers from some uncertainties. The time which elapses from the sampling operation till the washing of mitochondria can hardly be reduced to less than 10 sec, and this extra incubation results in an uptake of radioactivity, as shown by the fact that when atractyloside is added to mitochondria before filtration, less radioactivity is incorporated. Moreover, one may wonder whether the spreading of mitochondria on the filter modifies the structure of their membranes and influences their permeability.

The centrifugal layer filtration (27–29) which permits incubation periods of the order of a few seconds is elegant in principle. However, it was not found to be totally satisfactory (see also Winkler et al. (10)), especially because of uncertainties about the behavior of mitochondria at the interfaces between the different layers in the centrifuge tube. The only way to evaluate the incubation time is by standardization with another enzymatic reaction. Kleingberg, Pfaff, and Kröger (28) used the formation of acetocetate from \( \beta \)-hydroxybutyrate as a standard reaction; however, the use of this reaction as a standard method raises some problems such as the permeability of mitochondria to \( \beta \)-hydroxybutyrate and acetocetate, the possible retention of acetocetate within mitochondria, and the sensitivity of the
acetoacetate determination. We have preferred to use the incorporation of \(^{32}P\)-phosphate into mitochondrial adenine nucleotides by oxidative phosphorylation for the standardization of incubation periods.

The results obtained by the centrifugal layer filtration method were in good agreement with those obtained by the atractyloside-centrifugation method. The standard centrifugation method gave somewhat higher values of incorporation. Since the atractyloside-centrifugation method gave the same results as the centrifugal layer filtration technique but was more convenient for routine assays, its use was preferred in most of the experiments on nucleotide exchange described in this paper.

The impurities contained in \((\text{14C})\)-CDP, \((\text{14C})\)-GDP, and \((\text{14C})\)-UDP, were detected by two-dimensional chromatography on Whatman No. 1 paper. The first development was made with isobutyric acid-1 m ammonia-0.4 m EDTA (200:120:1, v/v) (Reference 30). After drying in air the second development was made with 1-butanol-acetic acid-water (2:1:1) as the solvent.

**TABLE I**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Time</th>
<th>Intranitochondrial adenine nucleotides</th>
<th>Outofmitochondrial adenine nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(^{14}C)+(^{12}C) ((\text{AMP}+\text{ADP}+\text{ATP}))</td>
<td>(^{14}C) ((\text{AMP}+\text{ADP}+\text{ATP}))</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Direct exchange</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>155</td>
<td>0</td>
</tr>
<tr>
<td>((\text{14C}))-ADP</td>
<td>2</td>
<td>150</td>
<td>37</td>
</tr>
<tr>
<td>((\text{14C}))-ADP</td>
<td>30</td>
<td>154</td>
<td>94</td>
</tr>
<tr>
<td>Reverse exchange</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>210</td>
<td>108</td>
</tr>
<tr>
<td>((\text{14C}))-ADP</td>
<td>30</td>
<td>206</td>
<td>106</td>
</tr>
<tr>
<td>((\text{14C}))-ADP</td>
<td>5</td>
<td>200</td>
<td>51</td>
</tr>
<tr>
<td>((\text{14C}))-ADP</td>
<td>15</td>
<td>215</td>
<td>41</td>
</tr>
<tr>
<td>((\text{14C}))-ADP</td>
<td>30</td>
<td>205</td>
<td>37</td>
</tr>
</tbody>
</table>

\(^{14}C\)-Cytidine, \(^{14}C\)-guanosine, and \(^{14}C\)-uridine nucleotides were prepared free of any adenine nucleotide contaminant by paper chromatography using Whatman No. 1 paper and the phosphate-ammonium sulfate-propanol solvent prepared by adding 600 g of \((\text{NH}_4)\)SO\(_4\) and 20 ml of 1-propanol to 1 liter of 0.1 \(\mu\) sodium phosphate, pH 6.8. The separation was 20 hours at 20°. The purification was carried out on the \((\text{14C})\)-nucleotides diluted by \((\text{12C})\)-nucleotides. After elution with water, the salts were eliminated by passing the nucleotide solution over charcoal. The nucleotides were thereafter eluted from charcoal with ammonia-ethanol-water (1:50:49, v/v) as described by Hurlbert (31). The compounds purified according to this procedure contained over 93% of their radioactivity as NDP; the remainder contained NMP and NTP, but no detectable \((\text{14C})\)-adenine nucleotides.

**RESULTS**

**Conditions Required for Maintenance of Stable Pool of Internal Adenine Nucleotides during Transmembrane Exchange**—Mitochondria incubated at 0° in a medium supplemented with EDTA and \((\text{14C})\)-ADP incorporate radioactivity (Table I, Experiment 1). Conversely, mitochondria loaded with \((\text{14C})\)-adenine nucleotides by preliminary incubation with \((\text{14C})\)-ADP (see "Materials and Methods") lose their radioactive adenine nucleotides to the external medium provided this medium is supplemented with unlabeled ADP (Table I, Experiment 2). In both cases, the total amount of internal adenine nucleotides does not vary, indicating that ADP added to mitochondria is exchanged with the internal adenine nucleotides. Preparations of mitochondria used in this work contained from 10 to 15 \(\mu\)moles of adenine nucleotides and from 1.7 to 2.3 mg of water per mg of protein (determinations on 40 and 7 mitochondrial preparations, respectively). From these data it can be calculated that the ratio of concentrations of internal adenine nucleotides to external adenine nucleotides in this experiment is 1.9 to 2.1 mg of water. The above results strongly suggest that in this experiment the added ADP has been translocated into mitochondria by an exchange-diffusion process.

Although these results are in agreement with previous reports (2–13), it must be pointed out that an exchange between internal and external adenine nucleotides without alteration of the concentration of internal adenine nucleotides is achieved only when the incubation is carried out at 0° in the presence of EDTA. In fact, a loss of mitochondrial adenine nucleotides to the external medium (Table I) is observed at temperatures higher than 0°; this loss is substantially increased by the addition of \(\text{MgCl}_2\) or inorganic phosphate or both. At 0°, \(\text{MgCl}_2\) itself induces, as shown in the following paper (32), a redistribution of the internal adenine nucleotides in the mitochondrial spaces. Furthermore, as shown below, when \(\text{MgCl}_2\) is added together with ADP to a suspension of mitochondria, the added ADP is immediately converted by transphosphorylation into AMP and ATP. For these reasons, it was preferred to assay the adenine nucleotide translocation at 0° and in the presence of EDTA. When tested under these conditions, the translocation of external ADP is characterized by an initial rapid phase of incorporation of the added \((\text{14C})\)-ADP for about 1 min followed by a period of slower incorporation (Fig. 1A).

It is known that \(\text{MgCl}_2\) potentiates the inhibitory effect of atractyloside on the adenine nucleotide translocation (5). Owing to the presence of EDTA in the medium, a relatively high con-
centration of atractyloside was needed to stop the translocation reaction in the atractyloside-centrifugation method described above. Data in Fig. 2 illustrate the variation of the inhibition caused by atractyloside upon the incorporation of ($^4$C)-ADP when the concentration of atractyloside is increased. In the

TABLE II

Effect of adenine nucleotides on the transmembrane exchange of adenine nucleotides

Each assay tube contained $10 \, \text{mm}$ succinate (Experiments 1 and 3) or glutamate (Experiment 2), $100 \, \text{mm}$ KCl, $8 \, \text{mm}$ Tris-HCl buffer, and where indicated $16 \, \text{mm}$ potassium or sodium phosphate, $6 \, \text{mm} \text{MgCl}_2$, or $1 \, \text{mm} \text{EDTA}$. The total volume was $4 \, \text{ml}$ and the final pH was 7.4. Incubation was started by the addition of mitochondria (16 mg of protein) were incubated for 1 min at $0 \, \degree \text{C}$ in KCl-Tris-EDTA (see “Materials and Methods”) with $0.25 \, \text{mM}$ ($^4$C)-ADP and atractyloside as indicated. The pH was 7.4 and the total volume, 2.2 ml. Incubation was stopped by the standard centrifugation method. Without atractyloside (control taken as 100%), mitochondria incorporated 26 mmoles of ($^4$C)-ADP.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mitochondrial protein</th>
<th>Temperature</th>
<th>Period of incubation</th>
<th>Additions</th>
<th>Adenine nucleotide released</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>mg</td>
<td>0°</td>
<td>0</td>
<td>None</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>28</td>
<td>4</td>
<td>EDTA</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>28</td>
<td>4</td>
<td>Mg++</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>28</td>
<td>4</td>
<td>P$_i$ + EDTA</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>28</td>
<td>4</td>
<td>P$_i$ + Mg++</td>
<td>112</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>0°</td>
<td>0</td>
<td>None</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
<td>15</td>
<td>P$_i$ + Mg++</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>15</td>
<td>15</td>
<td>P$_i$ + Mg++</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>15</td>
<td>15</td>
<td>P$_i$ + Mg++</td>
<td>115</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>0°</td>
<td>0</td>
<td>None</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>P$_i$ + Mg++</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>P$_i$ + EDTA</td>
<td>10</td>
</tr>
</tbody>
</table>

Fig. 1. A, time variation of ($^4$C)-ADP incorporation during the transmembrane exchange of adenine nucleotides. Mitochondria (24 mg of protein) were incubated at $0 \, \degree \text{C}$ in the KCl-Tris-EDTA medium (see “Materials and Methods”) with 0.25 mm ($^4$C)-ADP. The pH was 7.4 and the total volume, 4.2 ml. Incubation was stopped by the atractyloside-centrifugation method; $n_i$ is the experimental equilibrium determined as indicated in the text (B). B, determination of the experimental equilibrium. The reciprocal of the ($^4$C)-ADP incorporation (data of A) has been plotted against the reciprocal of the incubation time.

Fig. 2. Effect of atractyloside on the transmembrane exchange of adenine nucleotides. Mitochondria (16 mg of protein) were incubated for 1 min at $0 \, \degree \text{C}$ in KCl-Tris-EDTA (see “Materials and Methods”) with 0.25 mm ($^4$C)-ADP and atractyloside as indicated. The pH was 7.4 and the total volume, 2.2 ml. Incubation was stopped by the standard centrifugation method. Without atractyloside (control taken as 100%), mitochondria incorporated 26 mmoles of ($^4$C)-ADP.

In a previous paper (3), the theoretical equilibrium was calculated as $(N_s \cdot N_f)/(N_s + N_f)$ where $N_s$ is the amount of added ($^4$C)-ADP and $N_f$ the amount of mitochondrial adenine nucleotides as determined on an aliquot fraction of the mitochondrial suspension. Subsequently, it was found that in the suspensions of mitochondria made in isotonic sucrose, a small but significant amount of the so-called mitochondrial adenine nucleotides is al-
TABLE III
Specific activity of mitochondrial adenine nucleotides during successive exchanges with AOPCP

<table>
<thead>
<tr>
<th>No. of incubations with AOPCP</th>
<th>Intramitochondrial adenine nucleotides</th>
<th>Labeling ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(AMP + ADP + ATP)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>([12C + 14C]</td>
<td>(AMP + ADP + ATP)</td>
</tr>
<tr>
<td></td>
<td>mg mols</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AMP</td>
<td>ADP</td>
</tr>
<tr>
<td>0</td>
<td>4090</td>
<td>1880</td>
</tr>
<tr>
<td>1</td>
<td>3570</td>
<td>1660</td>
</tr>
<tr>
<td>2</td>
<td>3200</td>
<td>1470</td>
</tr>
<tr>
<td>3</td>
<td>2950</td>
<td>1310</td>
</tr>
<tr>
<td>4</td>
<td>2690</td>
<td>1200</td>
</tr>
<tr>
<td>5</td>
<td>2370</td>
<td>1080</td>
</tr>
<tr>
<td>6</td>
<td>2140</td>
<td>950</td>
</tr>
<tr>
<td>7</td>
<td>1900</td>
<td>860</td>
</tr>
<tr>
<td>8</td>
<td>1640</td>
<td>770</td>
</tr>
<tr>
<td>9</td>
<td>1430</td>
<td>670</td>
</tr>
</tbody>
</table>

Use of AOPCP to detect any compartmentation of internal adenine nucleotides is based on the following rationale. When mitochondria are incubated with (14C)-adenine nucleotides, only the noncompartmented fraction of internal adenine nucleotides will become labeled by exchange and the compartmented fraction will remain unlabeled. Conversely, when these (14C)-labeled mitochondria are incubated with unlabeled adenine nucleotides, the most easily exchangeable fraction will be the labeled one. Since AOPCP is differentiated from AMP, ADP, and ATP by enzymatic assays, the amount of compartmentalized adenine nucleotides within mitochondria previously loaded with (14C)-adenine nucleotides can be easily measured by following the specific radioactivity of internal adenine nucleotides during exchange with the added AOPCP. Any compartmentalization should lead to the maintenance of a segregated pool of (14C)-adenine nucleotides within labeled mitochondria, and to a decrease of the specific radioactivity of internal adenine nucleotides during exchange with external AOPCP. On the contrary, a constant specific radioactivity should indicate a complete exchangeability of internal adenine nucleotides.

The experiment was performed as follows. Rat liver mitochondria loaded with (14C)-ADP (see "Materials and Methods") were incubated with AOPCP and then sedimented by centrifugation. The mitochondrial pellet was resuspended and incubated in a fresh medium supplemented with AOPCP and again sedimented. This process was carried out nine times. As described in the legend of Table III, we estimated the sum of both total and labeled AMP, ADP, and ATP remaining within the mitochondrial particles after each step of incubation with AOPCP. The term "labeling ratio" (see Table III) refers to the ratio of the sum of labeled AMP + ADP + ATP to the sum of labeled and unlabeled AMP + ADP + ATP. As shown in Table III the labeling ratio remained essentially constant during the nine successive incubation steps, a result which strongly indicates that all adenine nucleotides present within mitochondria are exchangeable with external adenine nucleotides.

Variation of Rate of Exchange with Period of Incubation—As illustrated in Fig. 1A, the time course of (14C)-ADP incorporation into rat liver mitochondria is characterized by an initial rapid uptake of radioactivity which lasts for about 1 min at 0° followed by a much slower incorporation. Winkler et al. (10) have also emphasized the initial jump phenomenon in (14C) incorporation. On the other hand, Klingenberg and Pfaff (6) have reported that the ADP translocation follows second order kinetics. More recently, the same authors have shown (12) that the exchange following pseudo first order kinetics with respect to the concentration of endogenous ADP and ATP only and second order kinetics with respect to the concentration of total endogenous adenine nucleotides. By contrast we could not obtain an entirely satisfactory fit of our experimental kinetic data to either a second order or a first order reaction.

A different approach to the kinetics of (14C)-ADP incorporation was used by evaluating the mean rate of exchange during limited time intervals. For this purpose, the (14C)-ADP incorporation was approximated for convenience by an exponential process; the mean rate of exchange between times t1 and t2 was calculated by using Equation 1.

\[ v(t_2 - t_1) = \frac{1}{t_2 - t_1} \ln \left( \frac{1 - \frac{n_0}{n_i}}{1 - \frac{n_0}{n_f}} \right) \]
In this equation $v^{t_1-t_2}$ is the mean rate of exchange during the period $t_1 \to t_2$, $n_i^{t_1}$, $n_i^{t_2}$, and $n_i^{\infty}$ are the amount of (°C)-adenine nucleotides incorporated at times $t_1$, $t_2$, and at equilibrium, respectively. Equation 1 was derived from the expression given by Boyer, Luchsinger, and Falcone (33) for the evaluation of the rate of an exponential process.

The values which have been obtained upon analyzing the data of Fig. 1 by means of Equation 1 have been plotted in graph of Fig. 3. As shown in Fig. 3, the rate of exchange at 0° decreases abruptly during the first 1 min of incubation. This is followed by a marked and transitory increase of the exchange rate which culminates at 2 min and afterward by a new decrease. This apparent oscillatory fluctuation is a reproducible phenomenon; it may be related to the oscillations of the amount of ~P bonds in the internal adenine nucleotides observed after mitochondria were added to a medium deprived of oxidizable substrate and inorganic phosphate (Fig. 4); this medium was similar to that used for the study of ADP translocation, except that ADP was omitted.

Effect of External ADP Concentration on Rate of Exchange—
The first experiments on the affinity of the nucleotide translocation system in rat liver mitochondria led to $K_m$ values of 50 to 30 μM (6, 10) for ADP or ATP. More recent data by Klingenberg and Pfaff (12) gave $K_m$ values of 4 and 10 μM for ADP and ATP, respectively. Furthermore, Winkler and Lehninger (34) have reported a value of 1 μM for the dissociation constant of the complex formed between ADP and its binding site in mitochondrial membranes. In spite of their differences, these data point to the very high affinity of mitochondria for ADP or ATP.

In the experiment which is reported here (Fig. 5), we have measured the affinity of the translocation system for external ADP with added MgCl₂ or EDTA. The effect of varying the volume of the incubation medium on the rate of translocation was estimated when keeping constant the amounts of (°C)-ADP and of mitochondria. In this manner, both (°C)-ADP and mitochondrial concentrations vary by the same amount, and thus the (°C)-ADP incorporation reaches the same equilibrium value at any (°C)-ADP concentration. As shown in Fig. 6, the $K_m$ for ADP is 5.6 μM in the presence of EDTA, and 17 μM in the presence of MgCl₂. With the same conditions of incubation as for the determination, we have measured, after rapid filtration on Millipore filter (0.45 μm), the fraction of (°C)-ADP remaining as such in the medium outside mitochondria after a short incubation time with MgCl₂ or EDTA. After 7 sec of incubation with EDTA, 92% of the (°C)-ADP initially added is still present whereas after the same length of time with MgCl₂, more than...
The value of $Q_A$ for oxidative phosphorylation varies from 2 to 3 in the whole range of temperatures tested (0–20°C) with an activation energy of about 11 kcal, and that above 10°C the rates of oxidative phosphorylation and of adenine nucleotide exchange are of the same order of magnitude. One can explain the slowing down of the translocation rate above 10°C by assuming that external ADP is exchanged preferentially against internal ATP; in this case, the rate of exchange cannot be higher than the rate of ATP synthesis. Alternative explanations are that above 10°C, phase changes occur in phospholipids as described by Luzzati and Hudson (42), or that lipid-protein interactions are modified in mitochondrial membranes.

Our data on the temperature-dependence and activation energy of ADP exchange obtained directly from exchange experiments are in good agreement with some data published by Heldt and Klingenberg (9) who found an apparent activation energy of 34 kcal below 10°C and of 22 kcal above 10°C for the ADP-induced oxidation of reduced pyridine nucleotides, as well as for the rate of oxygen uptake by mitochondria in State 3. These ADP-stimulated reactions provided an indirect determination of the temperature-dependence of ADP translocation. However, more recently, Klingenberg and Pfaff (12) found in a direct determination of ADP exchange a single value of $Q_A$, 4.5, and of activation energy (28 kcal) between 1°C and 25°C; in their evaluation the exchange rate was calculated by taking into account only the sum of internal ADP and ATP, which are the most easily exchangesable adenine nucleotides. Nevertheless, their method applied to our data could not give a single value for $Q_A$ and activation energy between 0°C and 20°C. It is noteworthy that Haslam and Griffiths (43) recently reported a biphasic Arrhenius plot of the temperature-dependence of the rates of penetration of oxaloacetate and l-malate into rat liver mitochondria in the presence of ATP, with activation energies of about 48 kcal below 12°C and about 10 kcal above 12°C.

**Nucleotide Specificity**—As shown in Table IV (Experiment 1), mitochondria incubated with nonpurified (14C)-CDP, (14C)-GDP, or (14C)-UDP incorporated a low but significant amount of radioactivity which was not eliminated by repeated washings. This incorporation was found to be atractyloside-sensitive. For each type of nucleotide the amount which was incorporated into mitochondria was of the same order of magnitude as the amount already present within mitochondria (cf. data of Heldt and Klingenberg (44): CXP, 0.00; GXP, 0.23; UXp, 0.13 mmole per mg of protein). To ascertain that the observed incorporation was not due to a contamination by (14C)-adenine nucleotides, a careful analysis by paper chromatography of the different nucleotides tested was carried out. As can be seen from data in Table IV (Experiment 1), the maximum contamination of guanosine, cytidine, and uridine nucleotides by (14C)-adenine nucleotides was too small to explain the labeling of mitochondria only by incorporation of a (14C)-adenine nucleotide contaminant. It was therefore inferred that incorporation of (14C)-CDP (14C)-GDP, and (14C)-UDP arises by a transmembrane exchange with the internal homologous nucleotides. Nevertheless the possibility remained that the atractyloside-sensitive part of the incorporation could be due to the (14C)-adenine nucleotide contaminant. To test this possibility, the (14C)-nucleotides were freed of any adenine nucleotide contaminant, as described under “Materials and Methods." Mitochondria incubated with these purified nucleotides incorporated a detectable amount of radioactivity, but the sensitivity of the incorporation to atractyloside was lost (Table IV, Experiment...
2). Because of this loss of sensitivity to atractyloside, the meaning of nucleotide incorporation at zero incubation time is questioned; it may be assessed in terms of a rapid and limited binding of (\( ^{14}C \))-CDP, (\( ^{14}C \))-GDP, and (\( ^{12}C \))-UDP to mitochondrial membranes.

It has been reported that FeSO\(_4\) promotes the uptake of adenine nucleotides into mitochondria (7). The incorporation of CDP, GDP, and UDP into mitochondria is also substantially increased upon addition of FeSO\(_4\). As an example, mitochondria incubated at 0° with FeSO\(_4\) and (\( ^{14}C \))-CDP, (\( ^{14}C \))-GDP, and (\( ^{14}C \))-UDP under standard conditions as described under "Materials and Methods" accumulated in 2 min, 1.8, 0.7, and 0.5 mmoles of the respective nucleotides per mg of protein. Carafoli, Rossi, and Lehninger (45) have also described an accumulation of UTP in rat liver mitochondria upon addition of Ca\(^{2+}\).

It is noteworthy that although being incorporated to a small but significant extent in mitochondria, CDP, GDP, and UDP are unable to induce any release of radioactivity from mitochondria previously loaded with (\( ^{14}C \))-adenine nucleotides.

A new approach to the study of the specificity of nucleotide translocation in mitochondria arose from the observation that some methylene diphosphonic acid analogues of ADP and ATP (namely AOPCP, AOPOPCP, and AOPCPOP) are incorporated into mitochondria through an atractyloside-sensitive process (17). As shown in Fig. 7, when mitochondria loaded with (\( ^{14}C \))-adenine nucleotides are incubated with AOPCP, a release of radioactivity outside mitochondria occurs. This release is strongly inhibited by atractyloside. The effect of the added AOPCP on the release of internal adenine nucleotides recalls that of added ADP (see above results) and suggests that AOPCP has been incorporated and exchanged with internal adenine nucleotides. To prove it, it was necessary to show that the total amount of internal adenine nucleotides present in mitochondria (AMP + ADP + ATP + AOPCP) does not vary during incubation with AOPCP. Since AOPCP cannot be assayed enzymatically (17) the extent of AOPCP incorporation was measured as follows. Owing to the fact that AOPCP has the same extinction coefficient as ADP, the amount of mitochondrial adenine nucleotides present after incorporation of AOPCP (AOPCP + AMP + ADP + ATP) was determined by measuring the absorbance at 260 m\(\mu\). Because of this loss of sensitivity to atractyloside, the mean-
not AOPCP or AOPOPCP, but also dADP is exchanged when the concentration of dADP or AOPCP is increased to a low amount of ADP could compete with the exchange between released upon addition of ADP alone. It is striking that such a release of (4C)-adenine nucleotides induced by AOPCP or by dADP is inhibited by ADP. It is seen that 10 μmoles of ADP value 20 times higher than that of the added (4C)-ADP (Table VI, Experiment 1). Even ratios of GDP to ADP as high as 10 do not influence the rate or the extent of (4C)-ADP translocation is not the only factor governing the specificity of oxidative phosphorylation for ADP.

AOPCP and dADP are rapidly and extensively translocated into mitochondria like ADP and ATP via an atractyloside-inhibited process, they are unable to lower the incorporation of (4C)-adenine nucleotides induced by dADP or AOPCP by 10%. This is a minimal estimate which does not take into account the 4 μmoles of (4C)-adenine nucleotides released upon addition of ADP alone. It is striking that such a low amount of ADP could compete with the exchange between AOPCP or dADP and internal adenine nucleotides.

As shown also in Table VII, when AOPCP and dADP are

---

**TABLE V**

**Action of nucleoside diphosphates upon (4C)-ADP incorporation**

<table>
<thead>
<tr>
<th>Nucleotide added</th>
<th>2 min</th>
<th>15 min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>40</td>
<td>68</td>
<td>82</td>
</tr>
<tr>
<td>CDP</td>
<td>39</td>
<td>68</td>
<td>79</td>
</tr>
<tr>
<td>GDP</td>
<td>39</td>
<td>68</td>
<td>79</td>
</tr>
<tr>
<td>IDP</td>
<td>39</td>
<td>68</td>
<td>80</td>
</tr>
<tr>
<td>UDP</td>
<td>41</td>
<td>65</td>
<td>85</td>
</tr>
<tr>
<td>dADP</td>
<td>24</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>AOPCP</td>
<td>23</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>AOPOPCP</td>
<td>29</td>
<td>75</td>
<td>91</td>
</tr>
<tr>
<td>dADP + AOPCP</td>
<td>23</td>
<td>73</td>
<td>87</td>
</tr>
<tr>
<td>dADP + AOPOPCP</td>
<td>25</td>
<td>74</td>
<td>88</td>
</tr>
</tbody>
</table>

---

**TABLE VI**

**Action of nucleoside diphosphates upon (4C)-ADP incorporation**

Mitochondria (Experiment 1: 18 mg; Experiment 2: 25 mg; Experiment 3: 19 mg; Experiment 4: 24 mg) were incubated at 0° in 110 mM KCl, 20 mM Tris-HCl, 1 mM EDTA. The final pH was 7.4 and the total volume, 2.2 ml. Incubations were ended by the atractyloside-centrifugation method.

**References:**

1. Gregg (47) on sonic particles of rat liver mitochondria.
2. Pfaff and Klingenberg (11), we found that dADP added to mitochondria previously loaded with (4C)-adenine nucleotides is taken up by mitochondria and displaces an equivalent amount of (4C)-nucleotides. We also confirmed that added adenine and adenylate do not exchange with mitochondrial adenine nucleotides and that AMP is more slowly exchanged than ADP or ATP (11).

Pfaff and Klingenberg (11) have pointed to the existence of strong interactions between ADP and ATP during translocation. It is seen that the incorporation of (4C)-ATP into mitochondria is completely suppressed by equimolar unlabeled ATP (8) whereas the incorporation of (4C)-ADP is scarcely lowered by addition of equimolar unlabeled ATP. We found that GDP, GTP, and UTP are unable to interfere with the incorporation of (4C)-ADP (Table VI, Experiment 1). Even ratios of GDP to ADP as high as 10 do not influence the rate or the extent of (4C)-ADP incorporation (Table VI, Experiment 2). Similarly, adenine and adenylate do not interfere with the incorporation of (4C)-ADP.

Although dADP, AOPCP, and AOPOPCP are rapidly and extensively translocated into mitochondria like ADP and ATP via an atractyloside-inhibited process, they are unable to lower the incorporation of equimolar amount of ADP into mitochondria either by competition or by dilution (Table VI, Experiment 3). However, an inhibition of the (4C)-ADP translocation is observed when the concentration of dADP or AOPCP is increased to a value 20 times higher than that of the added (4C)-ADP (Table VI, Experiment 4). Conversely, as shown in Table VII, the release of (4C)-adenine nucleotides induced by AOPCP or by dADP is inhibited by ADP. It is seen that 10 μmoles of ADP lower the release of (4C)-adenine nucleotides by about 4 to 6 μmoles, i.e. by 10%. This is a minimal estimate which does not take into account the 4 μmoles of (4C)-adenine nucleotides released upon addition of ADP alone. It is striking that such a low amount of ADP could compete with the exchange between AOPCP or dADP and internal adenine nucleotides.

As shown also in Table VII, when AOPCP and dADP are
so selected as to maintain constant both the water content of
mitochondria and the amount of internal adenine nucleotides. Addition of EDTA and incubation at 0 ° were essential to the achievement of this condition. The ADP translocation then corresponds to a stoichiometric exchange across the mitochondrial membranes of one molecule of external ADP against one molecule of internal ADP or ATP, so that the concentration of internal adenine nucleotides remains constant.

**Kinetics**—The exchange of mitochondrial adenine nucleotides with added ADP cannot be considered as a single step reaction or as a reaction governed only by some simple factors as temperature and the concentrations of adenine nucleotides inside and outside mitochondria. If the translocation of the added ADP proceeded via an uncomplicated transmembrane exchange with internal adenine nucleotides, a constant rate of incorporation of ADP into mitochondria during the entire incubation period would be expected. This does not actually occur; instead, the rate of incorporation of external ADP undergoes oscillatory fluctuations, as determined by calculations on short time intervals.

Oscillation-like fluctuations of the amount of anhydride-bound phosphate groups in internal adenine nucleotides have also been detected when mitochondria are incubated aerobically in a medium deprived of phosphate, substrate, and ADP. This medium is similar to that used for exchange studies except that in the last case ADP is added to mitochondria. Comparison between both types of fluctuations suggests that the rate of adenine nucleotide translocation may well be dependent on the state of phosphorylation of the internal adenine nucleotides. It has been established (11), for instance, that internal AMP as such does not exchange with external ADP or ATP; it must be transphosphorylated into ADP prior to exchange. The progressive rate decrease of the (32P)-ADP incorporation after 3 to 5 min of incubation probably indicates that all the internal (32P)-ADP and (32P)-ATP has been exchanged and that the residual internal (32P)-AMP must be transphosphorylated into (32P)-ADP before participating in the exchange. As shown in the following paper, this transphosphorylation is at 0 ° the rate-limiting step in the overall process of exchange of the internal (32P)-AMP against the external (32P)-ADP.

Häfer and Pressman (49) reported oscillations of the amount of ~P bonds in mitochondrial adenine nucleotides during valinomycin-induced K+ transport. Oscillations of oxygen uptake, pyridine nucleotides reduction and water content were also observed by Packer, Utsumi, and Mustafa (50) and Utsumi and Packer (51) in mitochondria incubated in an hypotonic medium containing a permeant anion. Although our conditions of incubation are different from those used by these authors, the concomitant oscillatory fluctuations of the rate of adenine nucleotide exchange and of the amount of ~P bonds in mitochondria that we have observed may be associated with small fluctuations of the water or ions content in whole mitochondria or in mitochondrial spaces with consequent modifications of the permeability of mitochondrion to adenine nucleotides.

**Specificity**—As shown in this paper, minute but detectable amounts of CDP, GDP, and UDP are incorporated into mitochondria by an atractyloside-insensitive process. Any contamination of these nucleotides by adenine nucleotides has been ruled out. We did not try to verify whether the observed incorporation of CDP, GDP, and UDP corresponds to a net uptake or to an exchange with endogenous homologous nucleotides. However, since CDP, GDP, and UDP are unable to induce any release of (32P)-adenine nucleotides from labeled mitochondria, it

---

**Table VIII**

*Effect of phosphate on ~P bond level in internal adenine nucleotides at 0 °*

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>Additions</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th><del>P</del></th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>None</td>
<td>48</td>
<td>140</td>
<td>155</td>
<td></td>
</tr>
<tr>
<td>10 min</td>
<td>None</td>
<td>116</td>
<td>117</td>
<td>112</td>
<td>113</td>
</tr>
<tr>
<td>10 min</td>
<td>10 mM P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>196</td>
<td>73</td>
<td>70</td>
<td>229</td>
</tr>
</tbody>
</table>

---

**Table IX**

*Effect of phosphate on translocation of ADP and ADP analogues*

Mitochondria labeled with (32P)-adenine nucleotides (34 mg of protein containing 242 mmoles of (32P)-adenine nucleotide) were incubated at 0 ° for 7 min in 110 mM KCl, 20 mM Tris-HCl, pH 7.4. Incubation was started by the addition of mitochondria (30 mg of protein in 0.25 ml of 0.27 M sucrose, 1 mM Tris, pH 7.4) to 2 ml of 110 mM KCl, 20 mM Tris-HCl, pH 7.4. Incubation was carried out at 0 ° and stopped by addition of perchloric acid at a final concentration of 2%. ~P~ was calculated as the variation of the sum: 2 ATP + ADP.
can be concluded that the exchange, if any, does not occur at the expense of the mitochondrial adenine nucleotides.

It is noteworthy that intact rat liver mitochondria are able to incorporate added (14C)-UTP into mitochondrial RNA and that the incorporation is atractylloside-insensitive (52). It has also been shown (53) that mitochondria of Physarum polycephalum are able to carry out the synthesis of DNA (tested as the incorporation of (32P)-dADP), the synthesis being 90% inhibited in the absence of dGDP. Similar results have been recently reported for DNA synthesis in isolated rat liver mitochondria by Parsons and Simpson (54) and by Wintersberger (55) for isolated yeast mitochondria. These examples indicate that mitochondria may use external ribo- or deoxyribonucleotides for the synthesis of their own RNA or DNA. Surprisingly enough, although the structures of dADP and AOPOPCP are markedly different, their exchanges with intramitochondrial adenine nucleotides exhibit similar properties. Both are more atractylloside-sensitive than is the exchange of added ADP; both are inhibited by ADP and are stimulated by phosphate. Furthermore dADP and AOPOPCP scarcely affect the exchange of mitochondrial adenine nucleotides with added ADP. The results thus obtained with dADP and the phosphonic acid analogue of ADP point to a special role of adenine nucleotides in the exchange of mitochondrial adenine nucleotides with added ADP. The presence of adenine in a nucleoside di- or triphosphate is essential for it to be exchangeable with mitochondrial adenine nucleotides; in contrast, modifications of the ribose or of the phosphate groups of ADP and ATP only lowers the rate of exchange with mitochondrial adenine nucleotides without altering the atractylloside sensitivity of the exchange. Second, the ability of a nucleotide to take part in the reactions of oxidative phosphorylation in intact mitochondria is not restricted by its ability to exchange with mitochondrial adenine nucleotides as previously proposed by Pfaff, Klingenberg, and Heldt (2) and Kemp and Groot (16) on the basis of reports by L"ow et al. (48). Indeed, AOPOPCP and AOPOPCP (which are not substrates for reactions of oxidative phosphorylation) are rapidly exchanged with mitochondrial adenine nucleotides. Third, the exchange of dADP or AOPOPCP with mitochondrial adenine nucleotides is strikingly stimulated by the addition of phosphate. It is therefore logical to postulate, as did Pfaff and Klingenberg (11) for the exchange of external ADP, that added dADP and AOPOPCP preferentially exchange with internal ATP.

Acknowledgment—We wish to thank Mrs. Mireille Bof for excellent technical assistance.

REFERENCES

Kinetics and Specificity of the Adenine Nucleotide Translocation in Rat Liver Mitochondria
E. D. Duée and Pierre V. Vignais


Access the most updated version of this article at [http://www.jbc.org/content/244/14/3920](http://www.jbc.org/content/244/14/3920)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/244/14/3920.full.html#ref-list-1](http://www.jbc.org/content/244/14/3920.full.html#ref-list-1)