Kinetics of Phosphorylation of Intramitochondrial and Extramitochondrial Adenine Nucleotides As Related to Nucleotide Translocation*

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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 kinetics of oxidative phosphorylation of extramitochondrial (external) ADP and intramitochondrial (internal) ADP has been investigated under conditions required for the exchange-diffusion of adenine nucleotides through mitochondrial membranes (ethylenediaminetetraacetate-supplemented medium at 0°) and under conditions favoring the leakage of internal adenine nucleotides (Mg++-supplemented medium).

By means of atractyloside, it is possible to show that under conditions of exchange-diffusion the kinetics of phosphorylation of internal ADP and AMP are not altered by the addition of ADP to mitochondria and is independent of the phosphorylation of external ADP. Internal ADP and AMP are converted into ATP through a three-step process which successively involves first a rapid oxidative phosphorylation of the internal ADP and ATP, second a transient plateau phase, and finally a conversion of AMP into ATP. The oxidative phosphorylation of external ADP starts after a lag period which corresponds to the time interval required for the oxidative phosphorylation of the internal ADP into ATP.

The conversion of the internal AMP into ATP proceeds by two coupled reactions, namely an ATP-dependent transphosphorylation of AMP into ADP and the oxidative phosphorylation of ADP into ATP. As suggested by the data of 32P-labeling experiments, the enzymatic systems catalyzing the two above mentioned coupled reactions are structurally and functionally linked to form a complex which catalyzes the over-all conversion of AMP into ATP in a cyclic manner. The ATP which is the phosphate donor for the transphosphorylation reaction is not the free ATP pre-existing in mitochondria, but the ATP produced by the complex itself; on the other hand, the ADP formed as a transient intermediate in the AMP-ATP conversion is immediately phosphorylated to give ATP without mixing with the free internal ADP. These data point to a compartmentalization of the enzymatic complex involved in the AMP-ATP conversion.

Addition of magnesium ions to mitochondria incubated in a standard medium for oxidative phosphorylation (supplemented with O2, phosphate, and oxidizable substrate) results in a leakage of internal adenine nucleotides. This leakage is slowed by inhibitors or uncouplers of oxidative phosphorylation or by a low temperature, but not by atractyloside; ATP is preferentially released compared to ADP and AMP.

The adenine nucleotides released outside mitochondria are rapidly exchanged with those remaining inside mitochondria. Since the exchange is much faster than the release, the distribution patterns of the adenine nucleotides remaining inside mitochondria or released outside mitochondria are similar; for instance under the conditions of oxidative phosphorylation, the same high level of ATP is found inside and outside mitochondria. The effect of atractyloside on the apparent efficiency of the oxidative phosphorylation of internal adenine nucleotides is discussed with respect to the above data.

The evidence that adenine nucleotide translocation is the rate-limiting step in the oxidative phosphorylation of external ADP is based primarily upon the following experimental results:

(a) Atractyloside inhibits the phosphorylation of external (extramitochondrial) ADP, but it is without effect on the phosphorylation of internal (intramitochondrial) ADP (1-8). (b) Phosphorylation and translocation of external ADP are inhibited to the same extent by increasing amounts of atractyloside (4, 5). (c) Internal ADP is more rapidly phosphorylated than is external ADP (2, 5, 9, 10).

The experiments reported in this paper were designed to investigate more thoroughly the relationship between internal and external adenine nucleotides during the course of oxidative phosphorylation, and especially to evaluate by means of atractyloside the behavior of internal adenine nucleotides when external ADP is translocated into mitochondria and phosphorylated. The first part of this paper presents the kinetics of phosphorylation under conditions of strict exchange-diffusion of adenine nucleotides through mitochondrial membranes. The second part of the paper presents studies of phosphorylation under conditions of incubation which favor the leakage of internal
adenine nucleotides outside mitochondria. Under both sets of conditions, it was found that a small fraction of internal ADP and ATP does not react or reacts only very slowly in oxidative phosphorylation. These results are discussed with reference to the general properties of the adenine nucleotide translocation.

**METHODS**

Rat liver mitochondria were isolated according to the method of Hogeboom (11) in 0.25 M sucrose solution buffered at pH 7.3 to 7.4 by 1 mM Tris-HCl buffer. Potassium stratchylate (attractyloside) was extracted from rhizomes of the thistle *Atractylis gummifera* according to the procedure of Angelico (12) and recrystallized from 60% ethanol. Hexokinase (type V) was obtained from Sigma, (123) labeled ADP from Schwarz BioResearch, unlabeled nucleotides from P-L Biochemicals, phosphoenolpyruvate and the enzymes used for the determination of adenine nucleotides from Boehringer and Sons.

In experiments on oxidative phosphorylation, incubation was stopped by addition of perchloric acid at a final concentration of 2%. After neutralization of extracts, the amounts of AMP, ADP, and ATP were determined according to the method of Adam (13). The increase of phosphate bonds in adenine nucleotides (P - P) was determined as the increase of the sum: ADP + 2 ATP. Labeled AMP, ADP, and ATP arising from (14C)-ADP added to mitochondria were separated by paper chromatography (14) and located under ultraviolet light. Their respective areas were cut out from the paper strips for determination of their radioactivity by means of a thin window gas flow counter, model RA 15 (Intertechnique, Paris). When phosphorylation was carried out in the presence of (32P)-inorganic phosphate, the amount of 32P incorporated into ADP and ATP was determined after separation of ADP and ATP by two-dimensional paper chromatography (14, 15). The distribution of radioactivity between the respective amounts of AMP, ADP, and ATP was determined according to the method of Adam (13). The difference in the amount of each nucleotide present within the whole medium and the amounts found in the filtrate gives the amount of each nucleotide present within the mitochondrial particles.

When the phosphorylation pattern of adenine nucleotides outside mitochondria or inside mitochondia was to be determined, the incubation was ended as follows. An aliquot of the mitochondrial suspension was filtered through a Millipore filter (HAWP, 0.45 μ) fitted to a 5 ml multifit syringe with a Swinnny adapter. Filtrate, 1 ml, was recovered within 10 sec from each incubation medium and collected in tubes containing 0.1 ml of 2.5 N perchloric acid in order to inhibit immediately any enzymatic reaction. In an exactly parallel experiment, the incubation period was directly ended by addition of perchloric acid to the whole incubation medium. Adenine nucleotides were assayed in the perchloric extracts after neutralization by KOH according to the procedure of Adam (13). The difference between the respective amounts of AMP, ADP, and ATP found in the whole medium and the amounts found in the filtrate gives the amount of each nucleotide present within the mitochondrial particles.

In some experiments of this type, it was also necessary to determine the penetration and accumulation of inorganic phosphate in mitochondria. In this case three parallel identical incubations were carried out with a medium containing (32P)-phosphate. The first two were ended as described above for the determination of the phosphorylation pattern of internal and external adenine nucleotides. The third one was ended by a rapid centrifugation of mitochondria at 25,000 × g for 10 min at 0°C, the collection of mitochondria in a pellet being achieved in about 45 sec. The pellet was extracted with trichloracetic acid, and the total amount of phosphate present was estimated from the radioactivity of the trichloracetic acid extract assuming rapid equilibrium between internal and external inorganic phosphate. To calculate the exact amount of inorganic phosphate in mitochondria, it was necessary to subtract the (32P)-phosphate incorporated into ATP and ADP from the total amount of (32P)-phosphate (sum of organic and inorganic phosphate). Since dephosphorylation of ATP occurs in the pellet of mitochondria because of anaerobiosis, the amount of 32P incorporated into mitochondrial ATP and ADP had to be determined by using the data of the first two incubations.

The water content of mitochondria was estimated as follows. Mitochondria after incubation were isolated by centrifugation at 25,000 × g for 10 min. The tubes were blotted dry and immediately weighed. After drying overnight at 90°C, they were weighed again. The interparticular water in the pellet of mitochondria was estimated by including (13C)-dextran with a molecular weight of 60,000 to 90,000 and measuring the radioactivity present in the pellet. The pellets were dissolved with Hyamine and transferred to vials, and 20 ml of liquid scintillation fluid containing 0.4% of 2,5-diphenyloxazole and 0.03% of p-bis(2-(5-phenyloxazoly))benzene made up in toluene were added. The radioactivity was counted with a Nuclear-Chicago liquid scintillation counter.

Inner and outer membranes were prepared and purified according to the method of Parsons and Williams (16).

**RESULTS**

It has been recently reported (5, 17, 18) that rat liver mitochondria rapidly lose their adenine nucleotides without impairment of phosphorylation efficiency when they are incubated aerobically at room temperature with an oxidizable substrate, inorganic phosphate, and MgCl2. The leakage of adenine nucleotides is abolished when temperature is lowered to 0°C and when the medium is supplemented with EDTA (19). Thus, in order to fit the kinetics of phosphorylation of added ADP to a model of strict exchange-diffusion of adenine nucleotides through mitochondrial membranes, an EDTA-supplemented medium at 0°C is essential.

**Kinetics of Phosphorylation of External (14C)-ADP and of Internal (14C)-AMP and (14C)-ADP under Conditions of Exchange-Diffusion of Adenine Nucleotides—At first, we tried to evaluate, by means of attractyloside, the behavior of the pool of internal adenine nucleotides when external ADP is translocated and phosphorylated in mitochondria.

Two parallel series of incubation were carried out. The test tubes for both series contained the same basic components, i.e. inorganic phosphate, (12C)-ADP, and succinate, while the tubes of the second series also contained attractyloside. The phosphorylation of the internal (12C)-adenine nucleotides and of the added (12C)-ADP was followed as described under "Methods." Data on the formation of ~P in adenine nucleotides (Fig. 1, left) show that, as expected, attractyloside inhibits the phosphorylation of (12C)-ADP to about 83% but does not alter the kinetics of phosphorylation of the (12C)-adenine nucleotides. In the control experiment, during the 1st min of incubation, the amount of ~P bonds increases more rapidly in the (12C)-adenine nucleotides than in the (12C)-adenine nucleotides, which indicates in agreement with Heldt et al. (2, 10) that internal ADP is phosphorylated prior to external ADP. The initial
Translocation and Oxidative Phosphorylation

Fig. 1. Phosphorylation of internal adenine nucleotides and of added ADP. Mitochondria (34 mg of protein) were incubated at 0°C in 110 mM KCl, 14 mM Pi, 1 mM EDTA, 10 mM succinate, 0.36 mM (14C)-ADP. Atractylloside, where present, was 11 μM. The final pH was 7.4 and the total volume, 2.2 ml. Incubation was ended by addition of perchloric acid to a final concentration of 2%.

Fig. 2. Effect of the preliminary incubation period on the delayed phosphorylation of added ADP. Mitochondria (26 mg of protein) were added at zero time in 110 mM KCl, 20 mM Tris-HCl, 1 mM EDTA, 10 mM succinate, 0.23 mM (14C)-ADP. The final pH was 7.4 and the total volume, 2.3 ml. Temperature was 0°C. At the end of the preliminary incubation periods, inorganic phosphate was added (arrows) at a final concentration of 1.3 mM. Incubation was stopped by addition of perchloric acid to a final concentration of 2%.

The kinetic data of interconversion of (14C)- and (12C)-adenine nucleotides are illustrated in Fig. 1, middle and right, respectively. The rise in (14C)-ATP is stoichiometrically accounted for by the decrease of an equimolecular amount of (14C)-ADP (Fig. 1, middle). The small amount of (14C)-AMP which has accumulated during the first 5 min of incubation decreases steadily afterward. This transition occurs when the amount of (14C)-ADP has fallen to about one-third of its initial value; this suggests that (14C)-AMP is used for ATP synthesis only when the concentration of (14C)-ADP falls below a critical level. As shown in Fig. 1, right, the kinetics of conversion of (14C)-ADP and (12C)-AMP into (12C)-ATP is exactly the same in the presence or in the absence of atractylloside. Apparently translocation and phosphorylation of external ADP do not interfere with phosphorylation of internal ADP and AMP, as if external ADP on one hand and internal AMP and ADP on the other behave independently in phosphorylation reactions. The peculiar behavior of internal AMP may be explained either by nucleotide compartmentalization or by the rate-limiting reaction of transphosphorylation of AMP into ADP.

It is noteworthy that (12C)-ATP is synthesized during the first 2 min of incubation at the expense of only (12C)-ADP, whereas the level of (12C)-AMP remains constant during this period. It is obvious from this result, in contrast with a report by Ozawa (20), that ADP, not AMP, is the prime phosphate acceptor in oxidative phosphorylation (see also References 5 and 21). The transphosphorylation of AMP into ADP is evidently a preliminary step in the over-all conversion of AMP into ATP; the enzymes which are assumed to catalyze this transphosphorylation are Mg++-dependent (see "Discussion").

With respect to our finding that (14C)-EDTA does not enter the mitochondrial matrix at pH 7.4,1 it is inferred that the transphosphorylation of AMP into ADP is achieved in the matrix space, since in this space the concentration of Mg++ is twice as high as that of adenine nucleotides.

Effect of Preliminary Incubation Period on Delayed Phosphorylation of Added ADP—The lag phase in the phosphorylation of external ADP (see above experiment) which contrasts with the immediate phosphorylation of internal ADP has been investigated further (Fig. 2). Rat liver mitochondria are previously incubated under aerobicism in a medium supplemented with succinate and (12C)-ADP for different lengths of time. Although the medium is deprived of inorganic phosphate, (12C)-ADP is slowly phosphorylated by endogenous phosphate and the level of ~P in the (12C)-adenine nucleotides steadily increases. Addition of inorganic phosphate after different periods of preliminary incubation brings about a marked enhancement.

1 Unpublished data.
of the rate of phosphorylation of (\textsuperscript{14}C)-ADP. However, the degree of enhancement varies according to the length of the preliminary incubation period. Whereas phosphorylation is immediately stimulated to a maximum degree by addition of phosphate after 2 or 4 min of preliminary incubation, a lag period of 2 min is observed when phosphate is added together with (\textsuperscript{14}C)-ADP at zero time. The lag period is shortened to 1 min when phosphate is added after a preliminary incubation period of 1 min. The delay to obtain a maximal rate of (\textsuperscript{14}C)-ADP phosphorylation when (\textsuperscript{14}C)-ADP and phosphate are added together must be contrasted with the immediate and rapid translocation of (\textsuperscript{14}C)-ADP into mitochondria (19).

**Distribution Pattern of Internal and External Adenine Nucleotides during Course of Phosphorylation of Added ADP under Conditions of Exchange-Diffusion**—In order to investigate more thoroughly the relationships between the phosphorylation and the transmembrane exchange of adenine nucleotides under conditions of exchange-diffusion, we studied the kinetics of phosphorylation of adenine nucleotides present inside or outside mitochondria, by means of a typical phosphorylation experiment, carried out at 0\degree. In the presence of EDTA, phosphate, and succinate (Fig. 3, a and b). The procedure used to determine the internal and external adenine nucleotides after incubation is described under "Methods." It is seen that the initial accumulation of ATP at the expense of ADP is faster inside mitochondria than outside, a result which is in agreement with the rate-limiting effect of translocation in the phosphorylation of external ADP into ATP at 0\degree (2, 5, 10). The kinetics of ATP synthesis at the expense of both ADP and AMP within mitochondria is a typical three-step process (similar to that described in the above experiment, Fig. 1, right) which contrasts with the steady accumulation of ATP at the expense of ADP outside mitochondria, at least between the 2nd and the 10th min. From the 10th to the 15th min, the accumulation of ATP outside mitochondria has practically ended because of lack of external ADP; whereas AMP within mitochondria is slowly but continuously phosphorylated into ATP. Moreover, it is noteworthy that the percentage of AMP outside mitochondria remains negligible during the whole period of incubation, a finding which points to the inability of internal AMP to be exchanged with external ADP or ATP.

To assess the significance of the different distribution patterns of AMP, ADP, and ATP inside and outside mitochondria in terms of energy utilization, it was of interest to determine the free energy of hydrolysis of ATP in both spaces. For this purpose the amounts of inorganic phosphate having penetrated into mitochondria and the water content of mitochondria were determined as described under "Methods." As shown in Table I, the concentration of inorganic phosphate inside mitochondria remains practically constant from the 2nd to the 15th min of incubation, although the concentration of internal ATP has considerably increased at the expense of ADP and AMP. This result indicates that penetration of inorganic phosphate occurs continuously during the course of the incubation. The free energy of hydrolysis of internal and external ATP ($\Delta F'_{in}$ and $\Delta F'_{ex}$) has been calculated by taking the standard free energy of ATP hydrolysis ($\Delta F_{o}$) equal to $-7$ kcal per mole for internal ATP (23) (Mg$^{2+}$ present in the matrix space (22)) and to $-8.6$ kcal per mole for external ATP (23) (Mg$^{2+}$ absent outside mitochondria) (see legend of Table I). Although the ratio of ATP to (ADP $\times$ P$i$) is higher outside mitochondria than

### Table I

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Water content (\textsuperscript{14}C)</th>
<th>Intramitochondrial P$_i$ (mM)</th>
<th>Extramitochondrial P$_i$ (mM)</th>
<th>(ATP)$<em>{in}/(ADP)$</em>{in} \times (P_i)$_{in}$</th>
<th>(ATP)$<em>{ex}/(ADP)$</em>{ex} \times (P_i)$_{ex}$</th>
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<tbody>
<tr>
<td>2</td>
<td>33</td>
<td>12.0</td>
<td>12.0</td>
<td>93</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>41</td>
<td>12.0</td>
<td>12.0</td>
<td>211</td>
<td>107</td>
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<tr>
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<td>45</td>
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<td>12.7</td>
<td>1,905</td>
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</tr>
<tr>
<td>15</td>
<td>52</td>
<td>12.5</td>
<td>12.5</td>
<td>3,710</td>
<td>542</td>
</tr>
</tbody>
</table>

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**Fig. 3.** Distribution patterns of internal and external ATP, ADP, and AMP under conditions of exchange-diffusion of adenine nucleotides. Three parallel series of incubation were carried out at 0\degree. Each test tube contained 10 mM succinate, 100 mM KCl, 8 mM Tris-HCl, 12 mM (\textsuperscript{32}P)-phosphate, and 1 mM EDTA. The final volume was 4 ml and the final pH 7.4. Incubation was ended by high speed centrifugation and the pellet was assayed for water and radioactivity content as described under "Methods." In order to evaluate the amount of inorganic phosphate accumulated in mitochondria.
inside and outside mitochondria. Therefore, the above mentioned difference in the distribution patterns of external and internal adenine nucleotides may reflect only a difference in the concentration of the free and magnesium-bound forms of adenine nucleotides inside and outside mitochondria.

Distribution of Adenine Nucleotides Inside and Outside Mitochondria during Mg++-induced Leakage of Internal Adenine Nucleotides—In the experiments reported above, the pool of internal adenine nucleotides was maintained at a constant level in order to satisfy conditions of exchange-diffusion during the phosphorylation of external ADP. Experiments to be described now were on the contrary carried out under optimal conditions for specific leakage of internal adenine nucleotides (see previous paper). The temperature was raised to 28°C and the phosphorylation medium was supplemented with Mg++. The distribution patterns of ATP, ADP, and AMP released outside mitochondria or remaining inside mitochondria under different conditions of incubation are given in Table II.

In the absence of inhibitors or uncouplers, the distribution patterns of adenine nucleotides found inside and outside mitochondria are rather similar, although the percentage of external ATP is slightly higher than that of internal ATP and that, conversely, the percentage of external ADP is lower than that of internal ADP (Table II, Line 1). Data in Fig. 4 illustrate the time course of interconversion and leakage of internal adenine nucleotides in a control experiment carried out without inhibitors under conditions similar to those of Table II. Although the amounts of internal ATP, ADP, and AMP vary abruptly during incubation, their respective percentage remains the same from the 2nd to the 10th min of incubation. Calculations of the free energy of ATP hydrolysis from the data of Fig. 4 give about the same ΔF values for external and internal ATP (ranging from -10.5 to -11.2 kcal per mole), as was the case under conditions of exchange-diffusion. In this calculation, the standard free energy for ATP hydrolysis was taken equal to -7 kcal per mole for both internal and external ATP since in this case, Mg++ was added to the medium. Addition of oligomycin or 2,4-dinitrophenol leads to an extensive breakdown of ATP into ADP and AMP inside and outside mitochondria and also to a decreased rate of leakage of internal adenine nucleotides, in agreement with Meisner and Klingenberg (18) (Table II, Lines 2 and 3). Atractyloside does not prevent the net efflux of internal adenine nucleotides, but it markedly alters the phosphorylation pattern of the released adenine nucleotides favoring the accumulation of ADP and AMP at the expense of ATP outside mitochondria. However, it does not interfere with the phosphorylation of the adenine nucleotides remaining within mitochondria. This result is explained (see “Discussion”) by the fact that adenine nucleotides which have been released outside mitochondria behave as external adenine nucleotides toward mitochondria and are rapidly exchanged with the remaining internal adenine nucleotides. Actually the transmembrane exchange of adenine nucleotides measured at 28°C is at least 40 times faster than the Mg++-induced leakage of mitochondrial adenine nucleotides. Since atractyloside inhibits the transmembrane exchange but does not prevent the leakage of adenine nucleotides, it is easily understood that the ADP which has been released outside mitochondria cannot be replaced by ATP by means of exchange when atractyloside is present in the medium. The same rationale applies to the external AMP which is transphosphorylated into ADP by reaction with ATP through the action of adenylate kinase located in the outer compartment of mitochondria (24–27). Control

### Table II

<table>
<thead>
<tr>
<th>Additions</th>
<th>Adenine nucleotides released outside mitochondria</th>
<th>Adenine nucleotides remaining inside mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP</td>
<td>ADP</td>
</tr>
<tr>
<td>Total amount released (μm)</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>15 mM P₁ + 6 mM MgCl₂</td>
<td>64</td>
<td>16</td>
</tr>
<tr>
<td>15 mM P₁ + 6 mM MgCl₂ + 20 μg of oligomycin</td>
<td>50</td>
<td>22</td>
</tr>
<tr>
<td>15 mM P₁ + 6 mM MgCl₂ + 0.1 mM 2,4-dinitrophenol</td>
<td>34</td>
<td>22</td>
</tr>
<tr>
<td>15 mM P₁ + 6 mM MgCl₂ + 25 μM atractyloside</td>
<td>64</td>
<td>22</td>
</tr>
<tr>
<td>15 mM P₁ + 6 mM MgCl₂ + 25 μM atractyloside + 0.1 mM 2,4-dinitrophenol</td>
<td>41</td>
<td>3</td>
</tr>
</tbody>
</table>

Fig. 4. Distribution patterns of ATP, ADP, and AMP during the course of leakage of internal adenine nucleotides. Two parallel series of incubation were carried out at 28°C. Each test tube contained 100 mM KCl, 8 mM Tris-HCl, 12 mM P₁, 35 μM ADP, and 6 mM MgCl₂. The total volume was 4.2 ml and the final pH, 7.4. Incubation was started by adding to each tube 22 mg of mitochondrial protein in 0.25 ml of 0.25 mM sucrose. Incubation in the first series of tubes was ended by addition of 0.3 ml of 2.5 N perchloric acid and in the second series by filtration through Millipore filter as described under “Methods.”
experiments have actually confirmed that addition of AMP to mitochondria in a Mg++-supplemented medium results in accumulation of ATP outside mitochondria at the expense of the added AMP. If we consider the total amount of adenine nucleotides (those remaining inside mitochondria plus those released outside) in the absence or in the presence of atractyloside (Lines 1 and 4, Table II) the net effect of atractyloside is an apparent inhibition of ATP synthesis. This apparent inhibition is only observed when atractyloside is added to mitochondria under conditions of leakage and is explained, as stated above, by an inhibition of the exchange of the released AMP and ADP with the remaining internal ATP.

Addition of 2,4-dinitrophenol together with atractyloside (Table II, Line 5) results in a practically similar distribution of AMP, ADP, and ATP inside and outside mitochondria with a predominance of AMP in both spaces. This result indicates that AMP is easily released out of mitochondria upon addition of inorganic phosphate and Mg++ whereas it is slowly, if at all, exchanged with external adenine nucleotides (8). The Mg++-induced leakage of adenine nucleotides described here does not lead to large amplitude swelling nor to a decrease of the phosphorylation efficiency of mitochondria, in contrast to the un-
AMP into ATP, the first phase which lasts for 2 min being about five times faster than the second one. Atractyloside at a concentration of 20 mM inhibits the rapid initial conversion of AMP into ATP (Fig. 5, middle) and it simultaneously unMASKS the direct oxidative phosphorylation of ADP into ATP. The net result of the addition of atractyloside in the presence of Mg++ is an inhibition of the synthesis of ~P bonds in mitochondrial adenine nucleotides to an extent of about 40% (Fig. 5, right).

Entirely different results are obtained when MgCl2 is replaced by EDTA. In the presence of EDTA, the kinetics of phosphorylation resembles that already described for internal adenine nucleotides (see Fig. 1) where a typical plateau phase occurs after the initial rise in ATP synthesis but before the phosphorylation of AMP (Fig. 6, left); in this case atractyloside has no effect on the kinetics of phosphorylation of the internal AMP and ADP (Fig. 6, middle), nor on the increase of ~P bonds in adenine nucleotides (Fig. 6, right). It is clear that atractyloside does not alter the efficiency of oxidative phosphorylation in an EDTA-supplemented medium, whereas it apparently inhibits oxidative phosphorylation at 0° when the medium is supplemented with Mg++. As reported above (Table II) inhibition by atractyloside of the synthesis of ~P bonds in mitochondria incubated with a Mg++-supplemented medium at 28° is paralleled by a leakage of the internal adenine nucleotides. It was suggested that inhibition of oxidative phosphorylation of mitochondrial adenine nucleotides by atractyloside is only apparent and results from the fact that atractyloside does inhibit the exchange of the released ADP and AMP with the internal ATP but does not prevent the leakage of internal adenine nucleotides. The same explanation holds for the inhibitory effect of atractyloside in a Mg++-supplemented medium at 0° (see Fig. 5, right). However, it is noteworthy that the Mg++-induced leakage of internal adenine nucleotides is much less at 0° than at 28° (see previous paper) and that furthermore the outer membrane of mitochondria is readily permeable to adenine nucleotides (8). In keeping with the statement (8) that the adenine nucleotides translocate is located in the inner membrane of mitochondria and that atractyloside inhibits the adenine nucleotide translocation through the inner membrane, the above results may be interpreted by assuming that adenine nucleotides released out of the matrix space upon addition of Mg++ at 0° are partially retained in the intermembrane space by binding to the membranes which limit this space. This binding to the membranes of the intermembrane space would explain that internal adenine nucleotides in mitochondria incubated at 0° and in the presence of Mg++ are released outside the inner membrane without appearing in the extramitochondrial space.

Compartmentation of Enzymatic Complexes Involved in Conversion of AMP into ATP during Course of Oxidative Phosphorylation

As shown in Fig. 6, left, the incorporation of 32P into the P of internal ATP on the one hand and the net synthesis of internal ATP on the other follow parallel kinetics, a result which suggests that the ATP initially present in the preparation of mitochondria remains unalabeled. This ATP apparently escapes transphosphorylation reactions in contrast with the ATP newly generated by oxidative phosphorylation of ADP. Still more striking are the results reported in Fig. 7 which represent the kinetics of phosphorylation of mitochondrial adenine nucleotides at 28° in a Mg++-supplemented medium. In this case, although a large fraction of mitochondrial adenine nucleotides is released (50% at 5 min, 70% at 10 min), a constant amount of ATP still remains unlabeled, even after addition of ADP to mitochondria.

As typically illustrated in the case of the EDTA-supplemented medium (Fig. 6, left), after the initial rise in ATP there remains unphosphorylated a significant fraction of mitochondrial ADP amounting to nearly 20% of the total adenine nucleotides. This residual fraction of ADP is very slowly labeled by 32P, in contrast with the P of ATP. At the 6th min of incubation for instance, only 3 mmoles of 32P1 were incorporated into ATP, representing a 4% labeling of ADP, whereas 20 mmoles of 32P1 were incorporated into the P of ATP, representing a 12% labeling of the P of ATP. At the 6th min, the amounts of 32P1 incorporated into ADP and into the P of ATP were 6 and 37 mmoles, representing 11% and 19% of ADP and ATP, respectively. It is therefore obvious that the labeling of ADP is slower than the labeling of the P of ATP. All the above results lead to the following conclusions. (a) The (32P1)-ATP, i.e. the ATP newly generated by phosphorylation of ADP, is used for the transphosphorylation of AMP into ADP in preference to the (32P)-ATP present in mitochondria at zero time. (b) The (32P)-ADP formed through the above transphosphorylation does not mix with the residual (32P)-ADP which remains after the initial rapid phase of phosphorylation, but instead is immediately phosphorylated by 32P1 to give (32P2)-ATP. It must be recalled that the only substrate used in experiments reported in this paper has been...
FIG. 7. $^{32}$P incorporation into ATP and ADP under conditions of leakage of internal adenine nucleotides. Each test tube contained 10 mM succinate, 100 mM KCl, 8 mM Tris-HCl buffer, 12.5 mM ($^{32}$P)-phosphate, and 6 mM MgCl$_2$. The total volume was 4 ml and the final pH, 7.4. Incubation was started by the addition of 33 mg of mitochondrial protein in 0.3 ml of 0.25 M sucrose and was carried out at 28°C for the period of time indicated. After 5 min of incubation 85 mmoles of ADP were added to one tube and incubation was continued for 5 min. Dotted lines refer to phosphorylation in the presence of ADP. Numbers with asterisks refer to the ratio $^{32}$P$_2$/32P$_3$.

FIG. 8. Simultaneous exchange and leakage of adenine nucleotides.
be explained by a transitory inhibition of either the nucleoside diphosphokinase or the GTP-AMP phosphotransferase. In this respect it may be recalled that inhibition of the nucleoside diphosphokinase activity occurs at high concentrations of Mg-ADP and is counteracted by Mg-ATP (35, 36). Since the amount of Mg⁺⁺ in liver mitochondria (30 μmole per mg of protein) exceeds that of adenine nucleotides (10 to 15 μmole per mg of protein) (22) and because of the high affinity of Mg⁺⁺ for ADP and ATP (27), it appears that most of internal ATP and ADP are complexed with Mg⁺⁺. A dependence of the plateau phase on the ratio of internal Mg-ADP to internal Mg-ATP is therefore plausible.

Discrimination by Means of Atractyloside of Leakage and Exchange of Mitochondrial Adenine Nucleotides under Conditions of Oxidative Phosphorylation—Experiments described here showed that conditions which decrease the rate of ATP synthesis or which lead to a dephosphorylation of ATP also decrease the rate of leakage of internal adenine nucleotides; conversely, test conditions generally used for oxidative phosphorylation at room temperature are optimal for leakage of adenine nucleotides. Under these conditions, ATP is predominantly found outside mitochondria.

Atractyloside may be used as a means of differentiating between the exchange and the leakage of internal adenine nucleotides, since this compound inhibits the exchange but not the leakage. By means of atractyloside, it was shown here that the maintenance of a high level of ATP outside mitochondria is due to an unspecific leakage of internal adenine nucleotides superimposed upon a specific transmembrane exchange between ADP and ATP. This is illustrated by the scheme of Fig. 8; ADP and AMP which have leaked out of the matrix space are rapidly replaced in the external space (extramitochondrial and membrane space) by ATP. The external ADP is first exchanged with the internal ADP; it is therefore translocated into the matrix space and phosphorylated to give ATP. On the other hand, the ATP which has replaced ADP in the external space reacts there with AMP through the action of adenylate kinase to produce ADP. The newly generated ADP enters a new cycle of exchange with internal ATP across the inner mitochondrial membrane. By inhibiting the exchange of external ADP with internal ATP without altering the rate of leakage, atractyloside inhibits the accumulation of ATP outside mitochondria, but not the accumulation of ADP or of AMP.

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