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

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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°C 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 Km of the nucleotide-exchange system for extramitochondrial ADP is 5.6 μM in an EDTA-supplemented medium. It rises to 17 μM when EDTA is replaced by MgCl2.

The adenine nucleotide translocation exhibits a Q10 of 10 to 12 below 10°C and of 2 to 3 above 10°C. By contrast the Q10 of the phosphorylation of mitochondrial ADP is 2 to 3 within the temperature range 0–25°C.

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.

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 atracytylenigenin (7, 13), a property which has been used 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°C 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 (atracyloside) was extracted from the rhizomes of the thistle Atractylosis gummifera according to the method of Angelio (20) and recrystallized several times from
calculation of the sedimentation time is given in detail elsewhere. The bottom of the tube within a mean time of about 45 sec. The centrifugation conditions, mitochondria were collected at 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.

The phosphonic acid analogues of adenine nucleotides, adenosine 5'-methylene diphasphate, 5'-adenylylmethylene diphasphate (methylene bridge between P2 and P3), and adenosine 5'-methylene diphasphono-P3-phosphate (methylene bridge between P2 and P3), were purchased from Miles and Company, Inc., Elkhart, Indiana.

Mitochondria loaded with (32P)-adenine nucleotides were prepared as described previously (17). Rat liver mitochondria (final concentration, 15 mg per ml) were incubated at 0°C in 110 mM KCl, 20 mM Tris-Cl, pH 7.4, 1 mM EDTA, 0.25 mM (32P)-ADP; after 20 min of incubation, mitochondria were sedimented by a 10-min centrifugation at 10,000 × g. The sediment was washed by resuspension in 0.25 M sucrose and centrifugation. This washing procedure was repeated three times. After the third washing, mitochondria were finally suspended in 0.25 M sucrose.

In experiments on oxidative phosphorylation, incubation was stopped by addition of perchloric acid at a final concentration of 2%. After neutralization of the extract, the amounts of AMP, ADP, and ATP were determined by the methods of Adam (21); when phosphonate analogues were used, ATP was estimated by the method of Lamprecht and Trautschold (22) (see Reference 17). The increase of phosphate bonds in adenine nucleotides (ΔP), evaluated as the increase of labeled phosphate groups in adenine nucleotides, was calculated as the change of the sum: ADP + 2ATP. When (32P)-phosphate was used, adenine nucleotides labeled by 32P were extracted and estimated according to the method of Nielsen and Lehninger (23). Acetoacetate was determined by the method of Walker (24).

In experiments on the efflux of adenine nucleotides from mitochondria, incubation was stopped by centrifugation at 25,000 × g for 5 min. With this method, the distribution pattern of intramitochondrial adenine nucleotides cannot be attained as with the filtration method described previously (25); it was used, rather, to determine the experimental conditions leading to an efflux of adenine nucleotides from mitochondria, without regard to their distribution into AMP, ADP, and ATP. The supernatant fluid was acidified with perchloric acid (final concentration, 2%). After neutralization, AMP, ADP, and ATP were assayed enzymatically by the methods of Adam (21) with the use of a Chance-Aminco double-beam spectrophotometer, and their respective amounts were added together.

All the experiments on nucleotide translocation were carried out in the following basic medium: 110 mM KCl, 20 mM Tris-Cl buffer, pH 7.4, 1 mM EDTA, which will be referred to as KCl-Tris-EDTA; in the direct exchange process (32P)-ADP was added to this medium at concentrations usually ranging from 0.2 to 0.3 mM. An alternative method of incubation (reverse exchange) was to add unlabeled nucleotides to mitochondria previously loaded with (32P)-adenine nucleotides. Incubation was usually stopped by the addition of atracyloside at a final concentration of 10⁻⁴ M, immediately followed by centrifugation at 25,000 × 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 (26). As will be shown under “Results” (Fig. 2), 10⁻⁴ M atracyloside inhibits 75 to 80% of the exchange and higher concentrations of atracyloside 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, atracyloside was added prior to mitochondria and the centrifugation was started immediately after the addition of mitochondria. This method, here called the atracyloside-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 atracyloside, 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 (32P)-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 atracyloside-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 × g for 10 min without prior addition of atracyloside. The mitochondrial pellet was then washed and extracted as in the atracyloside-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.

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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 atracyloside 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. Klingenberg, Pfaff, and Kröger (28) used the formation of acetacetate from β-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 β-hydroxybutyrate and acetacetate, the possible retention of acetacetate within mitochondria, and the sensitivity of the
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Adenine Nucleotides during Transmembrane Exchange—Mitochondria incubated at 0° in a medium supplemented with EDTA and (14C)-ADP incorporate radioactivity (Table I, Experiment 1). Conversely, mitochondria loaded with (14C)-adenine nucleotides by preliminary incubation with (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 mmoles 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 ADP in Experiments 1 and 2 (Table I) ranges from 23 to 35. 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 MgCl₂ or inorganic phosphate or both. At 0°, MgCl₂ 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 MgCl₂ 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 (14C)-ADP for about 1 min followed by a period of slower incorporation (Fig. 1A).

It is known that MgCl₂ potentiates the inhibitory effect of atracyloside 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 (4C)-ADP when the concentration of atractyloside is increased. In the

![Table II](image)

**Table II**

*Efflux of adenine nucleotides from rat liver mitochondria*

Each assay tube contained 10 mM succinate (Experiments 1 and 3) or glutamate (Experiment 2), 100 mM KCl, 8 mM Tris-HCl buffer, and where indicated 16 mM potassium or sodium phosphate, 6 mM MgCl₂, or 1 mM EDTA. The total volume was 4 ml and the final pH was 7.4. Incubation was stopped by the atractyloside-centrifugation method; without atractyloside (control taken as 100%), mitochondria incorporated 20 mmoles of (4C)-ADP.

![Fig. 1](image)

**Fig. 1.** A, time variation of (4C)-ADP incorporation during the transmembrane exchange of adenine nucleotides. Mitochondria (24 mg of protein) were incubated at 0°C in the KCI-Tris-EDTA medium (see "Materials and Methods") with 0.25 mM (4C)-ADP. The pH was 7.4 and the total volume, 4.2 ml. Incubation was stopped by the atractyloside-centrifugation method; n₁ (w) is the experimental equilibrium determined as indicated in the inset (B). B, determination of the experimental equilibrium. The reciprocal of the (4C)-ADP incorporation (data of A) has been plotted against the reciprocal of the incubation time.

![Fig. 2](image)

**Fig. 2.** Effect of atractyloside on the transmembrane exchange of adenine nucleotides. Mitochondria (10 mg of protein) were incubated for 1 min at 0°C in KCl-Tris-EDTA (see "Materials and Methods") with 0.25 mM (4C)-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 20 mmoles of (4C)-ADP.

The reciprocal of the (4C)-ADP incorporation was plotted as a function of the reciprocal of the incubation period within mitochondria was 293 mmoles (Experiment 1), 342 mmoles (Experiment 2), and 365 mmoles (Experiment 3).
AOPCPOP, adenosine 5'-methylene diphosphono-P2-phosphate. Differentiated from AMP, ADP, and ATP by enzymatic assays. The rapidly exchanged with the internal adenine nucleotides by an ADP. By results obtained with AOPCP,1 a phosphonic acid analogue of were found to be exchangeable. This conclusion is corroborated by a much slower incorporation. Winkler et al. (10) have also emphasized the initial jump phenomenon in (\(^{14}\)C)-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 follows 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 (\(^{14}\)C)-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 (\(^{14}\)C)-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 follows 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 (\(^{14}\)C)-ADP incorporation was used by evaluating the mean rate of exchange during limited time intervals. For this purpose, the (\(^{14}\)C)-ADP incorporation was approximated for convenience by an exponential process; the mean rate of exchange between times \(t_1\) and \(t_2\) was calculated by using Equation 1.

![Equation](image)

### Table III

<table>
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<tr>
<th>No. of incubations with AOPCP</th>
<th>Intramitochondrial ((^{14})C)-(AMP + ADP + ATP)</th>
<th>Labeling ratio</th>
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<td>([\text{molecules}]^{\text{[14C]} + \text{[14C]}})</td>
<td>(\frac{(\text{AMP + ADP + ATP})}{(\text{AMP + ADP + ATP})})</td>
</tr>
<tr>
<td>0</td>
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<tr>
<td>9</td>
<td>1430</td>
<td>670</td>
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</table>

ways present outside the mitochondria through compartmentalization; this amount, \(n_o\), ranges from 5 to 15% according to preparations. Two corrections were therefore applied to our experimental results. First, the amount of the true intramitochondrial adenine nucleotides was taken equal to \(N_i - n_o\); second, the true specific radioactivity of the external (\(^{14}\)C)-ADP at zero incubation time was calculated as the specific activity of the added (\(^{14}\)C)-ADP multiplied by \((N_o)/(N_o + n_o)\). When the aforementioned corrections were made (graphical determination of \(n(o)\) (cf. Fig. 1B) and re-evaluation of the truly intramitochondrial adenine nucleotides), 96 to 100% of the intramitochondrial adenine nucleotides were found to be exchangeable. This conclusion is corroborated by results obtained with AOPCP;1 a phosphonic acid analogue of ADP.

As reported in a preliminary communication (17) AOPCP is rapidly exchanged with the internal adenine nucleotides by an atractyloside-sensitive process and, besides, it is easily differentiated from AMP, ADP, and ATP by enzymatic assays. The use of AOPCP to detect any compartmentation of internal adenine nucleotides is based on the following rationale. When mitochondria are incubated with (\(^{14}\)C)-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 (\(^{14}\)C)-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 (\(^{14}\)C)-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 (\(^{14}\)C)-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 (\(^{14}\)C)-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 (\(^{14}\)C)-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 (\(^{14}\)C)-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 follows 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 (\(^{14}\)C)-ADP incorporation was used by evaluating the mean rate of exchange during limited time intervals. For this purpose, the (\(^{14}\)C)-ADP incorporation was approximated for convenience by an exponential process; the mean rate of exchange between times \(t_1\) and \(t_2\) was calculated by using Equation 1.

![Equation](image)

1 The abbreviations used are: AOPCP, adenosine 5'-methylene diphosphonate; AOPOPCP, 5'-adenylylmethylene diphosphonate; AOPCPFOP, adenosine 5'-methylene diphosphono-P3-phosphate.
In this equation \( v_{t_{1}-t_{2}} \) is the mean rate of exchange during the period \( t_{1}-t_{2} \). \( n_{t_{1}} \), \( n_{t_{2}} \), \( n_{t_{3}} \), and \( n_{t_{4}} \) are the amount of \(^{14}\text{C}\)-adenine nucleotides incorporated at times \( t_{1} \), \( t_{2} \), \( t_{3} \), 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 \( 0^\circ \) decreases abruptly during the first \( \frac{1}{2} \) 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 \( \sim\text{P} \) 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 \( \mu \)M (6, 10) for ADP or ATP. More recent data by Klingenberg and Pfaff (12) gave \( K_{M} \) values of 4 and 10 \( \mu \)M for ADP and ATP, respectively. Furthermore, Winkler and Lehninger (34) have reported a value of 1 \( \mu \)M 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 MgCl\(_{2}\) 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 \(^{14}\text{C}\)-ADP and mitochondria. In this manner, both \(^{14}\text{C}\)-ADP and mitochondrial concentrations vary by the same amount, and thus the \(^{14}\text{C}\)-ADP incorporation reaches the same equilibrium value at any \(^{14}\text{C}\)-ADP concentration. As shown in Fig. 6, the \( K_{M} \) for ADP is 5.6 \( \mu \)M in the presence of EDTA, and 17 \( \mu \)M in the presence of MgCl\(_{2}\). With the same conditions of incubation as for the \( K_{M} \) determination, we have measured, after rapid filtration on Millipore filter (0.45 \( \mu \)m), the fraction of \(^{14}\text{C}\)-ADP remaining after the same length of time with MgCl\(_{2}\) or EDTA. After 7 sec of incubation with EDTA, 92% of the \(^{14}\text{C}\)-ADP initially added is still present whereas after the same length of time with MgCl\(_{2}\), more than 98% is still present.
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Effect of Temperature—Preliminary experiments showed that similar values of \(Q_10\) for adenine nucleotide exchange are obtained by both direct determination at 5 or 10 sec and by extrapolating to zero time. Therefore, for convenience, the values of \(Q_10\) were determined for the temperature range from 0° to 20° by using the rate of exchange at 5 sec as the basis.

The value of \(Q_10\) for oxidative phosphorylation varies from 2 to 3 in the whole range of temperatures tested (0–20°) with an activation energy of about 11 kcal, and that above 10° 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° 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°, 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° and of 22 kcal above 10° 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 \(Q_10\), 4.5, and of activation energy (28 kcal) between 1° and 20°; 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 \(Q_10\) and activation energy between 0° and 20°. 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° and about 10 kcal above 12°.

Nucleotide Specificity—As shown in Table IV (Experiment 1), mitochondria incubated with unpurified \(^{14}C\)-CDP, \(^{14}C\)-GDP, or \(^{14}C\)-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, 0.18 mumole per mg of protein). To ascertain that the observed incorporation was not due to a contamination by \(^{14}C\)-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 \(^{14}C\)-adenine nucleotides was too small to explain the labeling of mitochondria only by incorporation of a \(^{14}C\)-adenine nucleotide contaminant. It was therefore inferred that incorporation of \(^{14}C\)-CDP, \(^{14}C\)-GDP, and \(^{14}C\)-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 \(^{14}C\)-adenine nucleotide contaminant. To test this possibility, the \(^{14}C\)-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 (14C)-CDP, (14C)-GDP, and (14C)-UDP to mitochondrial membranes.

It has been reported that FeSO4 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 FeSO4. As an example, mitochondria incubated at 0°C with FeSO4 and (14C)-CDP, (14C)-GDP, and (14C)-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. Cardon, Rossi, and Lehninger (45) have also described an accumulation of UTP in rat liver mitochondria upon addition of Ca++. 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 (14C)-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 (14C)-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 μm of perchloric acid extracts of mitochondria. The absorbance at 260 μm was actually the same, independent of whether the mitochondria were incubated with AOPCP or with ADP. These data allow us to conclude that when AOPCP is added to mitochondria previously loaded with (14C)-adenine nucleotides, a definite amount of AOPCP is incorporated into mitochondria whereas an equal amount of adenine nucleotides is released outside mitochondria. The over-all process corresponds to a transmembrane exchange of external AOPCP with internal adenine nucleotides. Similar results were obtained when mitochondria were incubated with AOPOPCP or with AOPCPOP.

The fact that AOPCP and AOPOPCP are translocated into mitochondria is all the more noteworthy as AOPCP and AOPOPCP are very slowly, if at all, metabolized within mitochondria. It has been reported previously (17) that AOPOPCP is not a substrate for ATPase in digitonin particles prepared from rat liver mitochondria. As shown in Table V, AOPOPCP is a poor phosphate acceptor for oxidative phosphorylation. When β-hydroxybutyrate is oxidized by digitonin particles, AOPCP is phosphorylated much more slowly than ADP (4 as fast) and the phosphate to acetoacetate ratio is lowered by about 80%. Separation of ADP, ATP, AOPCP, and AOPOPCP by paper chromatography (46) revealed that the small amount of (32P)-phosphate esterified in the presence of AOPCP was actually present in AOPOPCP. Moreover, other nucleoside diphosphates (CDP, GDP, IDP, UDP) have been found not to be substrates for oxidative phosphorylation in digitonin particles from rat liver mitochondria. This result is in agreement (except for IDP) with the

![Fig. 7. Exchange of mitochondrial (14C)-adenine nucleotides (14C)-Ad. N.) with added ADP or AOPCP. Mitochondria were previously loaded with (14C)-adenine nucleotides, as described under "Materials and Methods." Mitochondria (19 mg) containing 110 mmoles of (14C)-adenine nucleotides were incubated at 0°C in 110 mM KCl, 20 mM Tris-HCl, 1 mM EDTA. Additions were either 0.23 mM ADP or 0.23 mM AOPCP; atractyloside, where present, was 23 μM. The final pH was 7.4 and the total volume, 2.2 ml. Incubation was stopped by the atractyloside-centrifugation method.

<table>
<thead>
<tr>
<th>(14C)-Nucleotide added</th>
<th>Amount added</th>
<th>Conversion by adenine nucleotides</th>
<th>14C incorporation</th>
<th>H2O</th>
<th>Without atractyloside</th>
<th>With atractyloside</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mmoles</td>
<td>mmoles</td>
<td>mmoles</td>
<td></td>
<td>2 min</td>
<td>30 min</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>600</td>
<td>15</td>
<td>40</td>
<td>81</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>CDP</td>
<td>600</td>
<td>0.7</td>
<td>0.9</td>
<td>1.8</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>GDP</td>
<td>600</td>
<td>1.5</td>
<td>2.5</td>
<td>5.9</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>UDP</td>
<td>600</td>
<td>0.1</td>
<td>1.4</td>
<td>2.7</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>540</td>
<td>22</td>
<td>49</td>
<td>145</td>
<td>33</td>
<td>95</td>
</tr>
<tr>
<td>CDP</td>
<td>440</td>
<td>0.0</td>
<td>3.5</td>
<td>4.7</td>
<td>5.9</td>
<td>4.9</td>
</tr>
<tr>
<td>GDP</td>
<td>350</td>
<td>0.0</td>
<td>4.4</td>
<td>5.7</td>
<td>5.9</td>
<td>5.5</td>
</tr>
<tr>
<td>UDP</td>
<td>300</td>
<td>0.0</td>
<td>2.5</td>
<td>3.5</td>
<td>4.1</td>
<td>3.4</td>
</tr>
</tbody>
</table>
results of Gregg (47) on sonic particles of rat liver mitochondria, but in contradiction with the results of Löwe, Vallin, and Alm (48) obtained with sonic particles of bovine heart mitochondria.

Not only AOPCP or AOPOPCP, but also dADP is exchanged with internal adenine nucleotides although there is no evidence for a direct phosphorylation of dADP (16). In agreement with

**Table V**

**Phosphate acceptor activity of nucleoside diphosphates in rat liver digitonin particles**

The basic reaction medium for both experiments contained 82 mM KCl, 15 mM Tris-HCl, 8.3 mM phosphate labeled with 32P, 18 mM β-hydroxybutyrate, and 4 mg per ml of bovine serum albumin. The final pH was 7.4. Incubations were carried out at 28°. In Experiment 1, digitonin particles (2.7 mg of protein) were incubated for 5 min in a final volume of 2.6 ml. In Experiment 2, digitonin particles (2.9 mg of protein) were incubated for 10 min in a final volume of 2.2 ml. Incubations were ended by the addition of 0.2 ml of 30% trichloracetic acid.

<table>
<thead>
<tr>
<th>Nucleotide added</th>
<th>Acetoacetate formed</th>
<th>ADP esterified</th>
<th>Ratio of -ADP to acetoacetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>70</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>1.5 mM ADP</td>
<td>180</td>
<td>100</td>
<td>0.77</td>
</tr>
<tr>
<td>1.5 mM AOPCP</td>
<td>70</td>
<td>12</td>
<td>0.14</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.8 mM ADP</td>
<td>230</td>
<td>310</td>
<td>1.35</td>
</tr>
<tr>
<td>4.3 mM CDP</td>
<td>100</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>4.5 mM GDP</td>
<td>160</td>
<td>13</td>
<td>0.08</td>
</tr>
<tr>
<td>4.8 mM GDP</td>
<td>150</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>4.4 mM UDP</td>
<td>150</td>
<td>14</td>
<td>0.00</td>
</tr>
</tbody>
</table>

**Table VI**

**Action of nucleoside diphosphates upon (14C)-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, 2.3 ml. Incubation was stopped after 15 min by the atractyloside-centrifugation method.

<table>
<thead>
<tr>
<th>Nucleotides added</th>
<th>(14C)-ADP Incorporation</th>
<th>(14C)-Adenine nucleotides released during incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 min</td>
<td>15 min</td>
</tr>
<tr>
<td></td>
<td>μmoles</td>
<td>μmoles</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>dADP (0.54)</td>
<td>40</td>
<td>34</td>
</tr>
<tr>
<td>AOPCP (0.54)</td>
<td>39</td>
<td>34</td>
</tr>
<tr>
<td>dADP (1.10)</td>
<td>49</td>
<td>45</td>
</tr>
<tr>
<td>AOPCP (1.10)</td>
<td>51</td>
<td>46</td>
</tr>
<tr>
<td>dADP (0.54) + AOPCP (0.54)</td>
<td>51</td>
<td>43</td>
</tr>
</tbody>
</table>

Pfaff and Klingenberg (11), we found that dADP added to mitochondria previously loaded with (14C)-adenine nucleotides is taken up by mitochondria and displaces an equivalent amount of (14C)-nucleotides. We also confirmed that added adenine and adenosine do not exchange with mitochondrial adenine nucleotides and that AMP is more slowly exchanged than ADP or ATP (11).

Data reported in this section clearly indicate that nucleotide translocation is not the only factor governing the specificity of oxidative phosphorylation for ADP.

**Nucleotide Interactions: Effect of Phosphate—Pfaff (8) and Pfaff and Klingenberg (11)—have pointed to the existence of strong interactions between ADP and ATP during translocation. For instance, the incorporation of (14C)-ATP into mitochondria is completely suppressed by equimolar unlabeled ADP (8) whereas the incorporation of (14C)-ADP is scarcely lowered by addition of equimolar unlabeled ATP. We found that CDP, GDP, and UDP are unable to interfere with the incorporation of (14C)-ADP (Table VI, Experiment 1). Even ratios of GDP to ADP as high as 10 do not influence the rate or the extent of (14C)-ADP incorporation (Table VI, Experiment 2). Similarly, adenine and adenosine do not interfere with the incorporation of (14C)-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 (14C)-ADP translocation is observed when the concentration of dADP or AOPCP is increased to a value 20 times higher than that of the added (14C)-ADP (Table VI, Experiment 4). Conversely, as shown in Table VII, the release of (14C)-adenine nucleotides induced by AOPCP or by dADP is inhibited by ADP. It is seen that 10 μmoles of ADP lower the release of (14C)-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 (14C)-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 (14C)-ADP incorporation after 3 to 5 min of incubation probably indicates that all the internal (14C)-ADP and (14C)-ATP has been exchanged and that the residual internal (14C)-AMP must be transphosphorylated into (14C)-ADP before participating in the exchange. As shown in the following paper, this transphosphorylation is at 0° the rate-limiting step in the over-all process of exchange of the internal (14C)-AMP against the external (14C)-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 a hypotonic medium containing a permant 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 mitochondria to adenine nucleotides.

**DISCUSSION**

Except for experiments concerning the effect of temperature on the rate of ADP translocation, experimental conditions were 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.

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It is noteworthy that intact rat liver mitochondria are able to incorporate added (3H)-UTP into mitochondrial RNA and that the incorporation is atractyloside-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 (3H)-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 AOPCP are markedly different, their exchanges with intramitochondrial adenine nucleotides exhibit similar properties. Both are more atractyloside-sensitive than is the exchange of added ADP; both are inhibited by ADP and are stimulated by phosphate. Furthermore dADP and AOPCP 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 the adenine moiety of the molecule. In fact, a number of adenine nucleotides tested (ADP, ATP, dADP, AOPCP, AOPOPCP) are exchanged with mitochondrial adenine nucleotides. Actually three conclusions can be drawn from the experiments with ADP analogues. First, 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 atractyloside 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 Löw et al. (48). Indeed, AOPCP and AOPOPCP (which are not substrates for reactions of oxidative phosphorylation) are rapidly exchanged with mitochondrial adenine nucleotides. Third, the exchange of dADP or AOPCP 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 AOPCP preferentially exchange with internal ATP. 

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REFERENCES

