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COMPARISON OF ADP AND ATP AS SUBSTRATES FOR THE ADENINE NUCLEOTIDE TRANSLOCATOR IN RAT-LIVER MITOCHONDRIA

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SUMMARY

1. The exchange of ATP with endogenous adenine nucleotides is biphasic in contrast to that of ADP and is dependent on the endogenous ATP/ADP ratio.

2. It is shown that the rapid initial phase of the ATP exchange is an electroneutral exchange of external ATP with internal ATP.

3. Uncoupler stimulates both the ATP and the ADP exchange.

4. Competition experiments show that the adenine nucleotide translocator has a high specificity for exogenous ADP compared to exogenous ATP. This affinity difference is more pronounced than that reported by Pfaff and Klingenberg (*Eur. J. Biochem.* (1968) 6, 66–79).

5. The competition between ATP and ADP for the translocator is dependent on the energy state of the mitochondria, the K_i for ATP increasing from 1.5 μM in the presence of uncoupler to 200 μM, under high-energy conditions, whereas the K_m for ADP is unaffected.

6. The K_m for ATP is also dependent on the energy state of the mitochondria, and is under both high- and low-energy conditions about the same as the K_i for ATP in the ADP exchange.

7. Since the energy state has no effect on the maximum velocity these effects on K_m reflect a change of K_D. The different K_D values for ATP and ADP in the controlled state explain the asymmetric behaviour of the adenine nucleotide translocator.

8. The influence of the energy state of the mitochondria on the K_i and K_m for ATP suggests the existence of different conformational states of the adenine nucleotide translocator.

INTRODUCTION

It has been shown that the inner membrane of intact rat-liver mitochondria contains a translocation system that transports adenine nucleotides[1,2] in a 1:1 exchange[3] across this membrane. A difference in reactivity between added ADP and ATP with this system was reported[4] earlier. The rate of exchange of endogenous

Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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adenine nucleotides is normally higher with exogenous ADP than with exogenous ATP, but uncoupler increases the rate with ATP to that with ADP[5]. This effect of uncoupler has been explained by Pfaff and Klingenberg[5] by its ability to facilitate the movement of charge-compensating protons. These authors also reported that ADP and ATP compete for entry into the mitochondrion.

In this paper the exchange kinetics and the competition between ADP and ATP under various conditions are further investigated.

METHODS

The exchange reaction in intact rat-liver mitochondria was studied by following the uptake or release of radioactive adenine nucleotides. The exchange is expressed as percentage of the value of uptake or release at isotopic equilibrium or as uptake or release of label (cpm/mg protein).

Forward exchange[5]

^{14}C- or ^{3}H-labeled nucleotides were added to a reaction medium containing rat-liver mitochondria and the uptake of radioactivity was measured.

Backward exchange[5]

Adenine nucleotides were added to an incubation medium containing prelabeled rat-liver mitochondria. Prelabeling was carried out by incubating the mitochondria for 1 h at 0 °C with ^{1}*I*C- or ^{3}H-labeled adenine nucleotides. After diluting 10 times with 0.25 M sucrose the mitochondria were spun down by centrifugation for 10 min at $10500+g$. The pellet was washed with 30 ml of 0.25 M sucrose and finally taken up in sucrose.

During the exchange reaction samples were taken and the mitochondria separated from the reaction mixture by rapid filtration according to Winkler *et al.*[6] or by rapid centrifugation as described by Pfaff *et al.*[5] using an Eppendorf microcentrifuge (type 3200).

Unless otherwise mentioned the reactions were performed in a vessel cooled to 0–2 °C in a cold-room (0–2 °C). Under these conditions we found that the kinetics were not as reported by Winkler *et al.*[6]. The standard incubation medium contained 75 mM sucrose, 15 mM KCl, 50 mM triethanolamine–KOH buffer, 4 mM EDTA, final pH 7.4.

It was necessary to treat the radioactive-labeled ATP solutions with phosphoenolpyruvate, pyruvate kinase and Mg^{2+} in order to eliminate contamination by traces of ADP with, because it is carrier-free, a high specific activity. The exchange reaction medium contained excess EDTA to remove the small amount of free Mg^{2+}, introduced by addition of the ATP solution.

ATP was determined with hexokinase (EC 2.7.1.1) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49), ADP with pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) as described by Bergmeyer[7]. A Zeiss spectrophotometer was used except for the determination of low concentrations of ADP or ATP when the more sensitive Aminco–Chance dual-wavelength spectrophotometer was used.

Rat-liver mitochondria were prepared by the method of Hoogeboom as described by Myers and Slater[8].

Protein was determined by the biuret method as described by Cleland and Slater[9].

Radioactivity was measured using a Nuclear Chicago liquid scintillation counter type ISOCAP 300. As scintillation liquid was used a mixture of toluene and 96% ethanol (19:6, v/v) containing 4 g 2,5-diphenyloxazole and 50 mg 1,4-bis-(5-phenyl-oxazolyl-2-)benzene per liter. 10 ml of this mixture was brought in a counting vial with a dried filter or 25 μl of the reaction mixture.

Calculation of v, $t_{\frac{1}{2}}$ and endogenous pool

The rate of exchange (v in nmoles·min^{-1}·mg protein^{-1}) can be expressed by the equation:

$$v = \frac{1}{t_{\frac{1}{2}}} \cdot \frac{EI}{E+I} \cdot \ln 2 \tag{1}$$

where $t_{\frac{1}{2}}$ (min) is the time required for 50% exchange and E and I (nmoles·mg protein^{-1}) the concentrations in the medium and matrix, respectively, of the nucleotides taking part in the exchange reaction. This expression, which is essentially the same as that reported by Pfaff *et al.*[10], is based on a model of a 1:1 exchange between two homogeneous pools in the medium and matrix, respectively.

The amount of radioactivity at time t in the I and E compartments (I_t* and E_t*, respectively) is given by Eqns 2 and 3, respectively.

$$I_t^* = \frac{IT^*}{E+I}(1 - e^{-[v(E+I)t]/EI}) \tag{2}$$

$$E_t^* = \frac{T^*}{E+I}(E + I\, e^{-[v(E+I)t]/EI}) \tag{3}$$

where T* is the amount of radioactivity added. From these equations, it follows that during a forward exchange, I_t* rises from zero to $IT^*/(E+I)$, whereas E_t* decreases from T* to $T^*E/(E+I)$. At isotopic equilibrium, no transport of label can be detected, since

$$\frac{T^*}{E+I} = \frac{I_\infty{}^*}{I} = \frac{E_\infty{}^*}{E} \tag{4}$$

The percentage exchange is given by $(I_t^*/I_\infty{}^*)$ 100 which yields

$$\ln (100 - \%\ \text{exchange}) = \ln \left[100\left(1 - \frac{I_t^*}{I_\infty{}^*}\right)\right] \tag{5}$$

Substituting the value for I_t* from Eqn 2 in Eqn 5 yields

$$\ln (100 - \%\ \text{exchange}) = \frac{-v(E+I)}{EI} + \ln 100 \tag{6}$$

By plotting ln (100−% exchange) against t, $t_{\frac{1}{2}}$ may be calculated.

From Eqn 4:

$$E = I \cdot \frac{E_\infty^*}{I_\infty^*}$$

$$= I \cdot \frac{T^* - I_\infty^*}{I_\infty^*} \quad (7)$$

The slope of the line relating E with $(T^* - I_\infty^*)/I_\infty^*$ gives the concentration of the exchangeable endogenous pool.

Chromatographic separation of the adenine nucleotides

Mitochondria were separated from the medium by rapid centrifugation through a silicone oil layer into 15% $HClO_4$. The distribution of the radioactivity over exogenous and endogenous AMP, ADP and ATP was determined by paper chromatography[11] using Whatman No. I paper. The eluent consisted of isobutyric acid–water–25% ammonia–0.1 M EDTA (62:34.6:3:1, by vol.; final pH 2–3). The R_F values were AMP, 0.51; ADP, 0.36 and ATP, 0.29.

To prevent salt effects from disturbing the chromatographic separation in the supernatant samples, the adenine nucleotides were first removed by adsorption on active charcoal and elution by a mixture of 96% ethanol–25% ammonia (1:1, v/v), according to Roos and Loos[12].

Phosphoenolpyruvate, pyruvate kinase, ADP and ATP were purchased from Boehringer and Söhne, the ^{14}C-labeled nucleotides from Calatomic, Los Angeles, U.S.A., and the ^{3}H-labeled nucleotides from The Radiochemical Centre, Amersham, England.

The membrane filters used were from Sartorius, 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazolyl-2-)benzene from Nenimy. Other chemicals were from British Drug Houses Ltd, or from Merck.

RESULTS

ADP and ATP as substrates for the translocator

It is shown above that an exchange of one molecule of exogenous ADP or ATP for one of endogenous ADP or ATP, measured by the uptake or release of radioactive adenine nucleotides, results in an exponential relation between the percentage exchange and the time. When ATP is added as exogenous substrate for the translocator this is denoted as an 'ATP exchange' and when ADP is added as an 'ADP exchange'.

Fig. 1 illustrates the exchange of endogenous nucleotides with labeled exogenous ADP or ATP. It can be seen that the ADP exchange is practically complete within 10 min, and that the uptake of ATP is much slower than that of ADP. This could be due either to a greater rate of ADP than ATP exchange, or to a smaller endogenous pool taking part in the ATP exchange than in the ADP exchange. The second possibility is excluded by the experiment shown in Fig. 2 from which it can be calculated that the sizes of endogenous pools are the same with ADP and ATP as exogenous substrate, at least at 20 °C (*cf.* ref. 13). The size of the endogenous pool equals that measured enzymatically. It may be concluded, then, that the ADP exchange is faster than the ATP exchange.

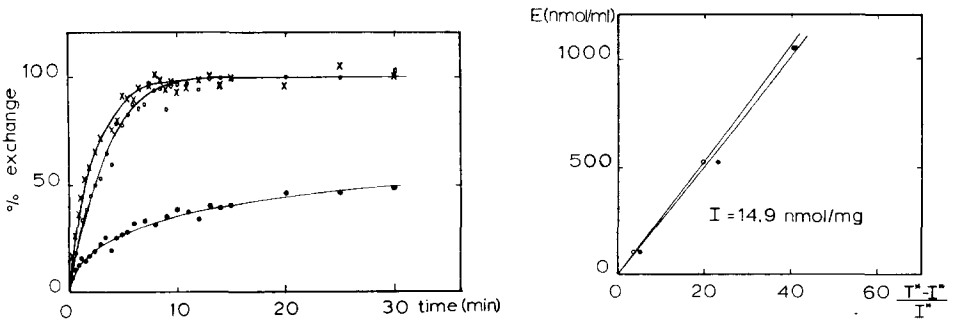


Fig. 1. Comparison of ADP and ATP as exogenous substrates for the exchange against endogenous adenine nucleotides. Rat-liver mitochondria were incubated in 4 ml standard reaction medium. The exchange was started by addition of 150 μM [^{14}C]ADP (52 cpm/nmole ○—○) or 150 μM [^{3}H]ATP (410 cpm/nmole) in the absence (●—●) or presence (× — ×) of 0.5 μM carbonyl cyanide *m*-chlorophenylhydrazone. Temperature 0 °C. 1.0 μg oligomycin/mg protein. Protein, 2.5 mg/ml. The exchange is expressed as percentage of the value reached at isotopic equilibrium with ADP.

Fig. 2. Size of the exchangeable endogenous pool as a function of the exogenous adenine nucletide concentration. Rat-liver mitochondria were incubated in 2 ml standard reaction medium. Various amounts of [^{3}H]ATP (124 cpm/nmole, ●—●) or [^{14}C]ADP (80 cpm/nmole, ○—○) were added as indicated on the ordinate. After 1 min at 20 °C the amount of radioactivity taken up (I^*) was determined. On the abscissa is plotted $(T^*-I^*)/I^*$ where T^* is the amount of radioactivity added. As indicated in Methods, the slope of these lines gives the size of the exchangeable pool of endogenous nucleotides. The enzymatically determined pool was 12.5 nmoles/mg protein.

In agreement with Pfaff and Klingenberg[5] we found that uncoupler greatly increases the rate of the ATP exchange (Fig. 1). However, in disagreement with Pfaff and Klingenberg, in our hands uncoupler also stimulates the ADP exchange[14] (Fig. 3) although the effect is much less pronounced than in case of the ATP exchange.

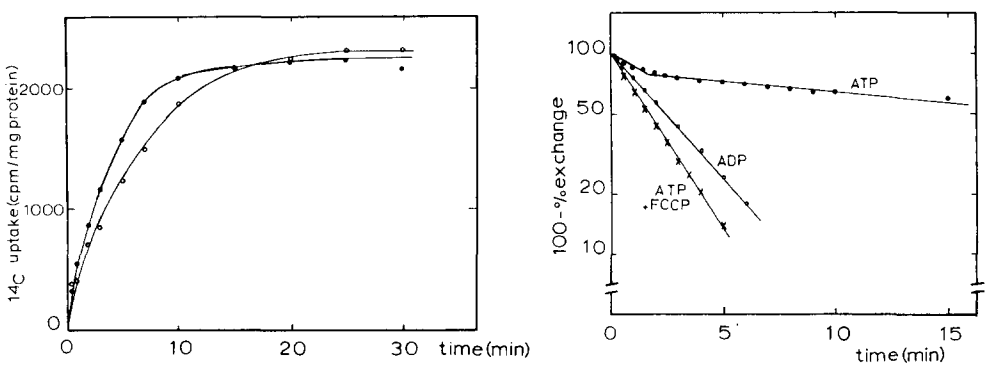


Fig. 3. Influence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) on the ADP exchange. Rat-liver mitochondria were incubated in 4 ml standard reaction medium, containing 10 μg oligomycin and 1.2 μg rotenone. After 4 min preincubation at 0 °C in the absence (○—○) or in the presence (●—●) of 0.5 μM FCCP, the exchange reaction was started by addition of 0.45 mM [^{14}C]ADP (134 cpm/nmole). Temperature, 0 °C. Protein 2.7 mg/ml.

Fig. 4. Kinetic behaviour of the ADP and ATP exchange. The results given in Fig. 1 are plotted logarithmically (see Methods).

In Fig. 1 can be seen that the ATP exchange shows heterogeneous kinetics, an initial rapid phase being followed by a slower. This biphasic character of the ATP exchange is clearer when the results given in Fig. 1 are plotted logarithmically (Fig. 4). In contrast to the ATP exchange in the absence of uncoupler the ATP exchange in the presence of uncoupler is homogeneous for at least 80% of the exchange. The percentage exchange at the end of the initial rapid phase of an ATP exchange corresponds to the percentage of ATP in the endogenous adenine nucleotides. This suggests that the initial rapid phase represents an electroneutral ATP_{out}–ATP_{in} exchange, whereas the slow phase may be an ATP_{out}–ADP_{in} exchange, hindered because of its electrogenic character (*cf.* ref. 5). This idea is confirmed by the following experiments.

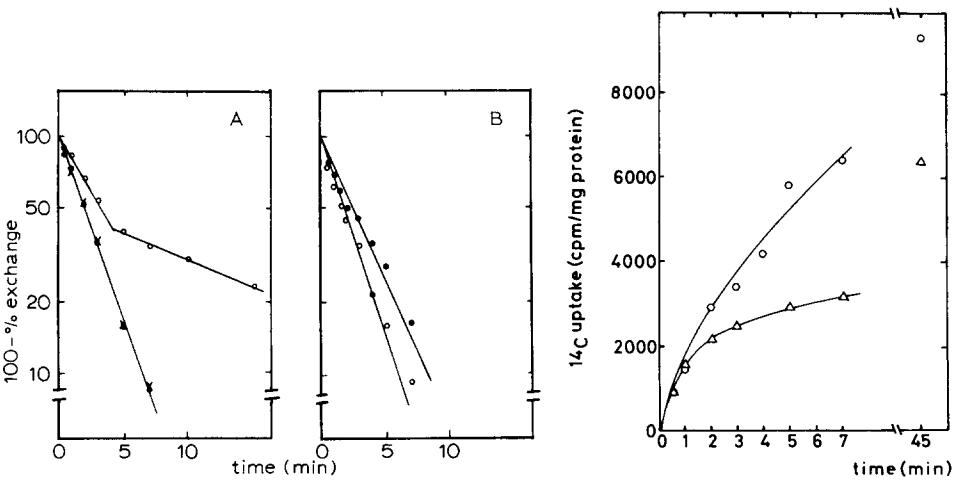


Fig. 5. The effect of preincubation of rat-liver mitochondria with succinate and phosphate on the ADP and ATP exchange. (A) Prelabeled mitochondria (1.2 mg/ml) were preincubated in 10 ml standard reaction medium in the absence or presence of 10 mM succinate, 10 mM phosphate and rotenone (1.6 μg/mg protein) for 3 min at 25 °C. After preincubation 5 μg oligomycin was added, the reaction mixture was cooled to 0 °C and the exchange was started by addition of 1 mM ATP or 1 mM ADP. ○—○, ATP exchange after preincubation in the absence of succinate and phosphate. ×—×, ATP exchange after preincubation with succinate and phosphate. ●—●, ADP exchange after preincubation in the absence of succinate and phosphate. (B) Mitochondria (3 mg/ml) were preincubated in 2 ml standard reaction medium in the absence (●—●) or presence (○—○) of 5 mM succinate and 4 mM phosphate for 3 min at 25 °C. After preincubation 5 μg of oligomycin was added, the reaction mixture cooled to 0 °C and exchange was started by addition of 300 μM [^{14}C]ADP.

Fig. 6. ATP exchange after preincubation of rat-liver mitochondria with succinate and phosphate in the presence and absence of oligomycin. Rat-liver mitochondria (6.6 mg protein) were preincubated in 2 ml standard reaction medium, containing 10 mM succinate, 10 mM phosphate, 1.5 μg rotenone, in the presence (△—△) or absence (○—○) of 10 μg oligomycin for 3 min at 25 °C. After preincubation 10 μg oligomycin was added when not already present. From this mixture 1.0 ml was used for the enzymatic determination of the endogenous adenine nucleotide pool. Another sample of 1.0 ml was added to 9.0 ml standard reaction mixture already cooled to 1 °C, and the exchange was started by addition of 260 μM [^{14}C]ATP (970 cpm/nmole). Protein during exchange reaction 0.33 mg/ml. The ATP content was 30% of the total amount of endogenous adenine nucleotides. The initial rapid phase extends to 25% of the total exchange being an uptake of 9200 cpm/mg protein in isotopic equilibrium.

When the endogenous ATP/ADP ratio is increased by preincubating the mitochondria in the presence of succinate and phosphate, the ATP exchange becomes homogeneous and as rapid as the ADP exchange (Fig. 5A). In Fig. 5B it is shown that this preincubation has only a small effect on the ADP exchange. That the effect on the ATP exchange is due to the high endogenous ATP content and not to the presence of succinate and phosphate *per se* is shown by the fact that preincubation with succinate and phosphate in the presence of oligomycin does not abolish the biphasic kinetics (Fig. 6). In Table I are summarized the endogenous ATP/ADP ratios after the various preincubation conditions.

TABLE I

INFLUENCE OF INCUBATION CONDITIONS ON THE ENDOGENOUS ATP/ADP RATIO

Prelabeled rat-liver mitochondria (2.1 mg/ml) were incubated in 2 ml of standard reaction mixture. Additions: oligomycin (3 μg/mg protein), 10 mM succinate, 10 mM phosphate or 0.5 μM FCCP. pH 7.4. After 3 min preincubation at 25 °C the mitochondria were separated from the medium by rapid centrifugation. The endogenous ATP/ADP ratio was determined by measuring the distribution of the radioactivity over the endogenous adenine nucleotides by the chromatographic procedure described in Methods.

Additions	$\frac{[ATP]_{end}}{[ADP]_{end}}$
Oligomycin	0.38
Oligomycin + succinate	0.22
Oligomycin + succinate + phosphate	0.35
Succinate + phosphate	2.74
Oligomycin + FCCP	0.30
FCCP	0.25

Direct measurements of the composition of the radioactively labeled exogenous and endogenous adenine nucleotide pools during an ATP and an ADP exchange after preincubation with succinate and phosphate are summarized in Table II. From the course of the exogenous and endogenous [^{14}C]ATP/[^{14}C]ADP ratios the conclusion is drawn that the mitochondria preferentially release ATP when exchanging with exogenous ATP.

Competition between ATP and ADP for the translocator

We have studied the influence of exogenously added ADP on the uptake of ATP and *vice versa*, by measuring the uptake of [^{3}H]ATP and [^{14}C]ADP simultaneously. The results are shown in Fig. 7A and Fig. 7B for the ^{14}C label and ^{3}H label incorporated, respectively. An equimolar amount of ATP has no effect on the uptake of ADP (Fig. 7A), whereas an equimolar amount of ADP strongly inhibits the uptake of ATP (Fig. 7B, *cf.* ref. 5). In the presence of uncoupler, however, an equimolar concentration of ATP inhibits ADP uptake, and an equimolar concentration of ADP inhibits ATP uptake, both by about 50%. Under these conditions, then, the affinities of ATP and ADP are about the same. The results presented in

TABLE II

RELEASE OF [^{14}C]ATP AND [^{14}C]ADP FROM PRELABELED MITOCHONDRIA DURING ATP AND ADP EXCHANGE

Prelabeled rat-liver mitochondria (1.2 mg/ml) were incubated for 3 min at 25 °C in 10 ml standard reaction mixture containing 10 mM succinate, 10 mM phosphate and rotenone (1 μg/mg protein). After the preincubation oligomycin (0.8 μg/mg protein) was added and the reaction mixture cooled to 0 °C. The exchange reaction was started by addition of 1 mM ATP or ADP. The reaction was stopped by rapid centrifugation. The distribution of radioactivity over ATP and ADP was measured chromatographically (see Methods).

Addition at zero time	*Time (min)*	$\frac{[^{14}C]ATP_{in}}{[^{14}C]ADP_{in}}$	$\frac{[^{14}C]ATP_{out}}{[^{14}C]ADP_{out}}$
1 mM ATP	0	2.4	—
	1	1.8	2.8
	2	1.7	—
	3	1.5	5.9
	4	1.2	7.1
1 mM ADP	0	1.5	—
	1	1.2	2.8
	2	1.1	1.7
	3	1.1	2.4
	4	1.0	2.2

Fig. 8 show that the effect of ATP on the rate of ADP uptake is dependent on the metabolic conditions. The inhibition is small under high-energy conditions (after preincubation with succinate *plus* oligomycin or succinate *plus* phosphate), more pronounced under low-energy conditions (absence of oxidizable substrate) and large in the presence of uncoupler.

Assuming that the inhibition is competitive[15] under all conditions, it is possible to determine K_i values from the equation:

$$\frac{V_{ADP}}{v_{ADP}} = 1 + \frac{K_{m\,ADP}}{[ADP]}\left(1 + \frac{[ATP]}{K_i}\right)$$

where V_{ADP} is the rate of uptake of ADP when $[ADP]_{out} \gg K_{m\,ADP}$ and $[ATP]=0$, v_{ADP} is rate of uptake of ADP in the presence of ATP, and $K_{m\,ADP}$ is about 1 μM (Fig. 9). Plotting V_{ADP}/v_{ADP} against [ATP] gives a straight line with slope $K_{m\,ADP}/K_i$ [ADP] from which the K_i of ATP for the inhibition of the uptake of ADP can be calculated (Fig. 10). After preincubation with succinate *plus* oligomycin or succinate *plus* phosphate a K_i of 100–200 μM can be calculated, while after preincubation without succinate this value is 5–10 μM and in the presence of uncoupler 1.5–3 μM.

K_m values of ATP and ADP for adenine nucleotide translocation under various energy conditions

From the Lineweaver–Burk plots given in Fig. 11 it is clear that the K_m value for ATP is also dependent on the energy state of the mitochondria being 1 μM, in the presence of uncoupler and 147 μM after preincubation with succinate and

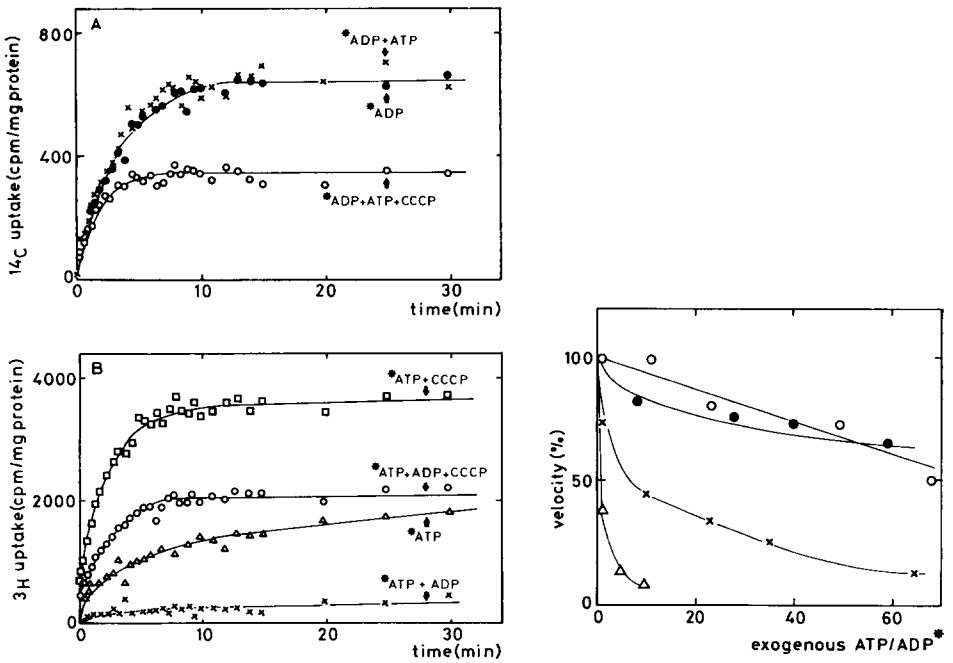


Fig. 7. The influence of ATP on the ADP exchange (A) and the influence of ADP on the ATP exchange (B). For incubation conditions see Fig. 1. A. Uptake of ^{14}C label. B. Uptake of ^{3}H label. ●—●, ADP exchange; △—△, ATP exchange; × — ×, exchange in the presence of equimolar amounts of ADP and ATP; □—□, ATP exchange in the presence of uncoupler (0.5 μM carbonyl cyanide *m*-chlorophenylhydrazone, CCCP); ○—○, exchange against equimolar amounts of ADP and ATP in the presence of uncoupler (0.5 μM CCCP).

Fig. 8. Influence of ATP on the ADP exchange after various preincubation conditions. Rat-liver mitochondria were preincubated for 3 min at 25 °C in 10 ml standard reaction medium, in the presence of 10 mM succinate and 10 mM phosphate (○—○), 10 mM succinate and 5 μg oligomycin (●—●), 5 μg oligomycin (× — ×) or FCCP (0.5 μM, △—△). Protein, 1 mg/ml. If not present during preincubation 5 μg oligomycin was added before starting the exchange reaction. The exchange was started by simultaneous addition of ATP and [^{14}C]ADP and measured at 0 °C. The ATP/ADP ratio was determined enzymatically.

phosphate. Under both conditions the V of the ATP uptake is about 2–3 nmoles/min per mg protein at 0 °C. From Fig. 10 and Fig. 11 it can be seen that the K_m value for ATP is about the same as the K_i value for ATP in an ADP exchange under various conditions. The K_m (1–2 μM) and the V for ADP (1–3 nmoles/min per mg protein at 0 °C (Fig. 9)) are independent of the energy state of the mitochondria.

DISCUSSION

The kinetics of the uptake of ADP by rat-liver mitochondria gives the theoretically expected result for a 1:1 exchange between two homogeneous pools, in contrast to the exchange against ATP which shows heterogeneous kinetics. The latter cannot be explained by the presence of AMP in the endogenous adenine nucleotide pool, since this would be seen also in the exchange against ADP[10]. Nor can it be explained

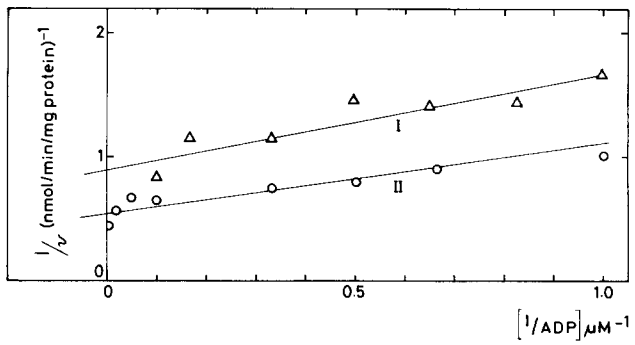


Fig. 9. Lineweaver–Burk plots of the velocity of ADP exchange as a function of the ADP concentration. I. Rat-liver mitochondria (1.5 mg protein) were preincubated in 3 ml standard reaction medium, containing 10 mM succinate, 10 mM phosphate and 1 μg rotenone for 3 min at 25 °C. After preincubation 10 μg oligomycin was added and 2.0 ml of this mixture was added to 48 ml standard reaction medium, already cooled to 0 °C. The exchange reaction was started by addition of labeled ADP (△—△). II. Rat-liver mitochondria (2.9 mg protein) were incubated in standard reaction medium, containing 1.5 μg rotenone and 10 μg oligomycin. The exchange reaction was started by addition of labeled ADP (○—○).

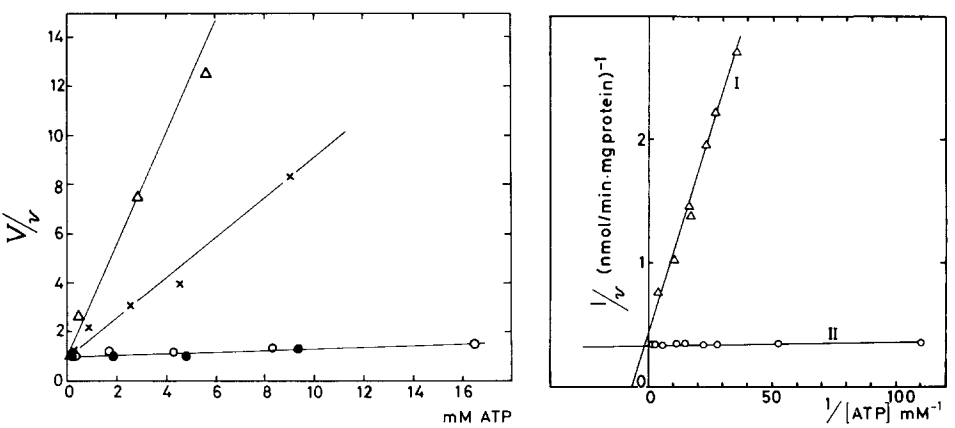


Fig. 10. Influence of ATP on V/v of the ADP exchange under various conditions. This figure is derived from the results presented in Fig. 8. From the slope of the lines K_i values can be calculated as is shown in Results. The symbols used are the same as in Fig. 8.

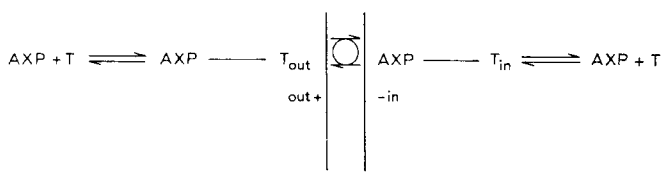
Fig. 11. Lineweaver–Burk plots of the velocity of ATP exchange as a function of the ATP concentration. I. Rat-liver mitochondria (3.8 mg protein) were preincubated in 2 ml standard reaction medium, containing 10 mM succinate and 10 mM phosphate and 5 μg rotenone, for 3 min at 25 °C. After preincubation 10 μg oligomycin was added and 1.0 ml of this mixture was added to 9.0 ml standard reaction medium, already cooled to 1 °C, and the exchange reaction was started by addition of labeled ATP in the concentrations indicated (△—△). II. Rat-liver mitochondria (3.8 mg protein) were brought into a standard reaction medium containing 10 μg oligomycin, 1.5 μg rotenone and 0.5 μM FCCP. After 3 min incubation at 0 °C the exchange reaction was started by addition of 1.81 μmole labeled ATP. By changing the volume of the reaction mixture the ATP concentrations as indicated were reached (○—○).

by a rapid aspecific binding of ATP to mitochondria, followed by a slow exchange of ATP against internal adenine nucleotides, since the initial rapid phase is seen both in a forward and a backward ATP exchange (Figs 4 and 5A). It is concluded then that the ATP exchange *per se* is heterogeneous and that the rapid phase is an electroneutral ATP_{in}–ATP_{out} exchange. This conclusion is supported by the findings that preincubation under conditions that give rise to a high endogenous ATP/ADP ratio results in a homogeneous exchange and by the preference for the release of endogenous ATP during an ATP exchange, carried out at a high endogenous ATP/ADP ratio. Under these conditions the ATP_{in}–ATP_{out} exchange is as rapid as the ADP exchange, which is presumably also electroneutral. Under conditions that give rise to an heterogeneous ATP exchange it is impossible even on the basis of a complete incorporation curve to calculate a reliable rate or K_m value for the ATP exchange.

With respect to the slow phase in the ATP exchange two possibilities are open. The slow phase could represent either an electrogenic ATP_{out}–ADP_{in} exchange, which is slow because it is inhibited by a membrane potential, or a redistribution of label from ATP_{in} *via* a leak through the oligomycin block and *via* the phosphotransferase reactions[16]. The existence of intramitochondrial reactions driven by added ATP, however, favours the idea that the slow phase is an ATP_{out}–ADP_{in} exchange since this exchange is necessary to provide endogenous ATP.

Pfaff and Klingenberg[5] demonstrated a competition between ADP and ATP for the translocator. Later studies by Klingenberg and co-workers[10,17–20] reported K_m values in the absence of Mg^{2+} for ADP ranging from 1.3 to 12 μM, and for ATP of 2.5–3 μM. In this paper, we demonstrate a large influence of the energy state of the mitochondria on the effect of ATP on the rate of uptake of ADP, inhibition by ATP being much less when the mitochondria are energy poor. The fact that preincubation with succinate *plus* oligomycin has the same influence on the K_i for ATP as preincubation with succinate *plus* phosphate, despite the different endogenous ATP/ADP ratio (see Table III), shows that the effect of ATP is regulated by the energy state of the mitochondria. In the presence of uncoupler, ATP and ADP serve equally well as substrates for the adenine nucleotide translocator.

The simplest representation of the process of adenine nucleotide translocation is:



Klingenberg and co-workers[15,17] report that the dissociation constants (K_D) of ADP–T_{out} and ATP–T_{out} are approximately the same. This leads them to the conclusion that the difference in behaviour of the adenine nucleotide translocator for exogenous ADP and ATP is not caused by a difference in dissociation constants but by difference in distribution of the ATP^{4-}- and ADP^{3-}-loaded translocator molecules over the mitochondrial inner membrane. Under the influence of a membrane potential (positive outside, negative inside) the ADP^{3-}-loaded carrier preferentially moves

TABLE III

SUMMARY OF BEHAVIOUR AND KINETIC CONSTANTS OF THE ADENINE NUCLEOTIDE TRANSLOCATOR UNDER VARIOUS ENERGY STATES

Preincubation conditions	*Energy state*	$\frac{[ATP]_{in}}{[ADP]_{in}}$	*ATP exchange*			*ADP exchange*			K_i *for ATP* (μM)
			Rate (*nmoles·min*$^{-1}$·*mg*$^{-1}$)	*Kinetics*	K_m (μM)	*Rate* (*nmoles·min*$^{-1}$·*mg*$^{-1}$)	*Kinetics*	K_m (μM)	
Succinate + phosphate	high	2.74	2–3	Homogeneous	147	2–3	Homogeneous	1–2	100–200
Succinate + oligomycin	high	0.22	—	Heterogeneous	—	2–3	Homogeneous	1–2	100–200
Oligomycin	low	0.38	—	Heterogeneous	—	2–3	Homogeneous	1–2	5–10
Uncoupler	low	0.25	2–3	Homogeneous	1	3–4	Homogeneous	1–2	1.5–3

to the inner side of the inner membrane while ATP^{4-}-loaded carrier stays on the outside. In this concept the structure of the translocator *per se* is not taken into account as the author states explicitly[18].

We agree that, under the low-energy conditions used by Weidemann *et al.*[15] the K_m values for ADP and ATP are about the same (1–3 μM).

We found, however, that the K_m for ATP_{out} is strongly dependent on the energy state of the mitochondria, whereas that for ADP is not influenced. The high K_m for ATP of 147 μM found under high-energy conditions is in agreement with the suggestion made previously that the State 3–State 4 transition is controlled by a competition between ATP and ADP for entry into the mitochondria[21].

The maximal velocities for ATP and ADP exchanges, are, however, approximately the same under various conditions, clearly showing that the difference in behaviour of the translocator for exogenous ATP and ADP in the controlled state is not caused by the distribution asymmetry of the loaded carriers proposed by Klingenberg and co-workers[15,17]. The different K_m values for ATP and ADP indicate that the cause of the affinity difference lies at the level of interaction of the adenine nucleotides with the carrier molecules. This means that the K_D values for exogenous ATP and ADP are not the same under energy-rich conditions.

The fact that Weidemann *et al.*[15] conclude that the dissociation constants for ADP and ATP are approximately equal and are not influenced by uncoupler is explained by the fact that they measured under low energy conditions, *i.e.* they used nucleotide-depleted mitochondria or they did their determination in the presence of arsenate.

The dependence of the K_m for ATP on the energy state of the mitochondria can be understood by assuming an effect on the conformation of the translocator protein or its environment. The existence of the translocator in different conformational states is in agreement with earlier suggestions of Vignais *et al.*[22], Leblanc and Clauser[23] and especially Vignais and Vignais[24].

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