Biochem. J. (1980) **192**, 75–83
Printed in Great Britain

The transport and accumulation of adenine nucleotides during mitochondrial biogenesis

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(*Received 19 February 1980/Accepted 30 May 1980*)

The atractyloside-insensitive accumulation of adenine nucleotides by rat liver mitochondria (as opposed to the exchange-diffusion catalysed by the adenine nucleotide translocase) has been measured by using the luciferin/luciferase assay as well as by measuring [^{14}C]ATP uptake. In foetal rat liver mitochondria ATP is accumulated more rapidly than ADP, whereas AMP is not taken up. The uptake of ATP occurs against a concentration gradient, and the rate of ATP uptake is greater in foetal than in adult rat liver mitochondria. The accumulated [^{14}C]ATP is shown to be present within the mitochondrial matrix space and is freely available to the adenine nucleotide translocase for exchange with ATP present in the external medium. The uptake is specific for ATP and ADP and is not inhibited by adenosine 5′-[$\beta\gamma$-imido]triphosphate, GTP, CTP, cyclic AMP or P_i, whereas dATP and AMP do inhibit ATP accumulation. The ATP accumulation is also inhibited by carbonyl cyanide *m*-chlorophenylhydrazone, KCN and mersalyl but is insensitive to atractyloside. The ATP uptake is concentration-dependent and exhibits Michaelis–Menten kinetics. The divalent cations Mg^{2+} and Ca^{2+} greatly enhance ATP accumulation, and the presence of hexokinase inhibits the uptake of ATP by foetal rat liver mitochondria. These latter effects provide an explanation for the low adenine nucleotide content of foetal rat liver mitochondria and the rapid increase that occurs in the mitochondrial adenine nucleotide concentration *in vivo* immediately after birth.

The exchange-diffusion transfer of ADP and ATP in mitochondria, which accounts for the uptake of ADP and for the simultaneous extrusion of ATP, has been studied in some considerable detail and is now reasonably well understood (for reviews see Klingenberg, 1976, 1979; Vignais, 1976, Vignais & Lauquin, 1979). On the other hand, there is virtually no information available on the mechanism by which mitochondria accumulate adenine nucleotides. Such a process is obviously of fundamental importance for the maintainence of a stable adenine nucleotide concentration within mitochondria, particularly in those cells and tissues in which mitochondrial biogenesis occurs at a significant rate. The uptake of adenine nucleotides would not only be required to maintain mitochondrial adenine nucleotide concentration for oxidative phosphorylation, but, together with other nucleotides, it would provide the building blocks for the synthesis of mitochondrial nucleic acids.

It has recently been shown that, during foetal and neonatal development of the rat, the adenine nucleotide content of hepatic mitochondria increases more than 2-fold within the first 2h after birth (Sutton & Pollak, 1978). Hence it was considered that foetal and neonatal rat liver mitochondria are especially suitable for the investigation of the mechanism that is responsible for the accumulation of nucleotides, and particularly adenine nucleotides, within the matrix space of mitochondria.

In an earlier study it was shown that the binding constants of foetal rat liver mitochondria for atractyloside did not differ significantly from those of adult rat liver mitochondria either in the presence or absence of ATP (Pollak *et al.*, 1978). The different kinetics of [^{14}C]ATP uptake and the incomplete inhibition of this uptake by atractyloside into foetal rat liver mitochondria suggested that these mitochondria possess an alternative mechanism for ATP transport in addition to the

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; p[NH]ppA, adenosine 5′-[$\beta\gamma$-imido]triphosphate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

0306-3283/80/100075-09$01.50/1

atractyloside-sensitive adenine nucleotide translocase (Sandor & Pollak, 1976; Pollak *et al.*, 1978). It has been proposed that it is this alternative atractyloside-insensitive mechanism that effects the accumulation of adenine nucleotides.

The aim of this study is to describe and characterize this transport mechanism in isolated rat liver mitochondria.

Experimental

Animals

Random-bred Wistar rats from the University animal house were used throughout this investigation. For experiments involving adult rat liver mitochondria, female Wistar rats weighing 150–200g were used. Foetal and suckling rats of known age were obtained from overnight (16h) matings.

Isolation of mitochondria

Mitochondria were isolated essentially as described previously (Pollak, 1975); the only modification involved an additional nuclear sedimentation (after the discontinuous-gradient nuclear separation) at $270g_{av.}$ for 10min in the high speed rotor (296) of the PR2 centrifuge, to ensure that the mitochondrial suspension was completely free from nuclei.

The isolation medium contained 250mM-sucrose, 1mM-Hepes, pH 7.4, 1mM-EDTA adjusted to pH 7.4 with NaOH and 0.2% bovine serum albumin.

Measurement of adenine nucleotide transport and accumulation

All incubations (except where especially stated) were carried out in 1.5ml Eppendorf 3810 plastic centrifuge tubes at 30°C in a DB3 Driblock Techne (Cambridge) heater. The volume of the incubation mixture was 1ml and contained 25mM-Hepes, pH7.4, 5mM-$MgCl_2$, 50mM-KCl and 0.2ml of isolation medium (see above), including the volume of the isolation medium in which the mitochondria were suspended. After the addition of the mitochondrial suspension (0.5–1.0mg of protein) the tubes were preincubated for 1min at 30°C before further additions were made as indicated in the text.

In the initial experiments, 10μl of 0.1M-ATP, ADP or AMP was added to commence the incubation. In subsequent experiments 10μl of 0.1M-[8-^{14}C]ATP or [8-^{14}C]ADP or [8-^{14}C]GTP was added as indicated. The ^{14}C-labelled nucleotides were all obtained from The Radiochemical Centre (Australia) Pty. and sufficient unlabelled carrier compound was added so that the 10μl that were added to the incubation mixture contained 1μmol of nucleotide and 0.1μCi of radioactivity (220d.p.m./nmol).

Assays of adenine nucleotide accumulation

At the end of the incubation period, 25μl of mersalyl (1mg/ml) was added to inhibit further transport (with the exception of the experiments described in Fig. 1) and the tubes were centrifuged in an Eppendorf 5412 centrifuge for 5min in the cold. The supernatant was aspirated, and the pellet was resuspended in 1ml of ice-cold incubation medium. After further centrifugation the supernatant was aspirated again and the tube containing the pellet was stored in liquid N_2.

Determination of adenine nucleotide uptake by the luciferase method. The stored pellets were resuspended on the same day in 500μl of water, in the cold. To 250μl of this suspension 25μl of 3M-$HClO_4$ were added and the mixture was placed into liquid N_2 until the total adenine nucleotide content of the mitochondria was determined by the luciferase method as described previously (Sutton & Pollak, 1978).

Determination of adenine nucleotide uptake by the isotope method. The stored pellets were resuspended on the same day in 200μl of water (there was no special need to keep the resuspended pellets cold) and a sample (100μl) was placed onto 2.5cm GF/C Whatman glass filter discs in scintillation vials and dried at 70°C. To the dried samples 10ml of scintillation fluid was added. The scintillation fluid consisted of 0.4% 2,5-diphenyloxazole in toluene (A.R. grade). All samples were counted in a model 2211 Packard liquid-scintillation spectrometer for at least 4000 counts and quench corrections were carried out with a quench curve in which acetone was used as a quenching agent.

The remaining portion of each sample that had been assayed either by the luciferase or the isotope method was stored at −30°C. Subsequently the protein concentration of these samples was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (fraction V; Sigma) as standard.

Results and discussion

Accumulation of ATP and ADP

When isolated foetal rat liver mitochondria were incubated in the presence of 1mM-ATP, -ADP or -AMP in a medium originally designed for the measurement of respiratory control of foetal rat liver mitochondria (though P_i was omitted) (Pollak, 1975), a time-dependent uptake of ATP and ADP was observed, but AMP was not taken up (Fig. 1). This uptake *in vitro*, which was measured by the direct determination of adenine nucleotides with the sensitive luciferase assay, is similar to the change that was observed *in vivo* within 2h of birth (2.62–6.03nmol/mg of protein) (Sutton & Pollak,

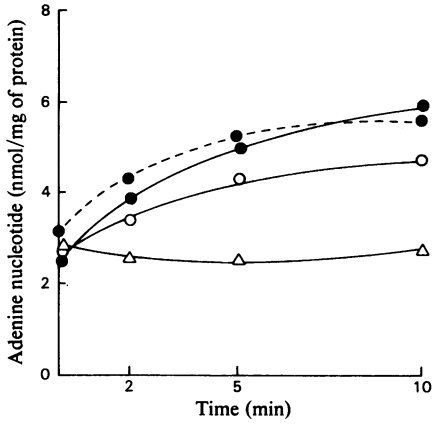

Fig. 1. *Accumulation of adenine nucleotides by foetal rat liver mitochondria*

Mitochondria isolated from 21 day foetal rat liver were incubated for up to 10 min at 30°C with 1 μmol of AMP, ADP or ATP. After the incubation, the mitochondria were washed and the total adenine nucleotide content was assayed with the luciferase method. Further details are given in the Experimental section. △, AMP; ○, ADP; ●, ATP; ●----●, ATP + atractyloside.

1978). The use of ^{14}C-labelled adenine nucleotides is more convenient but the method does not discriminate between the adenine nucleotide translocase activity, which will also contribute to the labelling of mitochondrial adenine nucleotides by the exchange-diffusion reaction, and the net accumulation of adenine nucleotides that is demonstrated in Fig. 1. Moreover potassium atractyloside is not an effective discriminatory inhibitor presumably since at 30°C and at the time intervals chosen to demonstrate optimally adenine nucleotide accumulation (0–10 min), it did not completely inhibit [^{14}C]ATP exchange (Table 1 and Fig. 1).

Comparison between the luciferase method and the isotope method

The assays in which [^{14}C]ATP was used to follow adenine nucleotide accumulation showed good agreement with the results obtained with the luciferase assays for the 2, 5 and 10 min samples of all the ages investigated. As expected the zero-time values obtained by the isotope assay reflected the incomplete exchange of the exogenous [^{14}C]ATP with the endogenous pool in the nominal zero-time tubes. This incomplete exchange is more marked in the mitochondria of suckling and adult rats. The apparent instantaneous exchange of most of the endogenous pool of foetal rat liver mitochondria has been reported before, when the adenine nucleotide

Table 1. *Accumulation of ATP by foetal rat liver mitochondria: comparison of the luciferase and isotope methods*

The data represent mean values ± S.E.M. for the number of experiments shown. Mitochondrial suspensions were incubated at 30°C with 1 μmol of [^{14}C]ATP, washed and both the total amount of adenine nucleotide and the radioactivity due to [^{14}C]ATP were assayed in portions taken from the same tube. Further details are given in the Experimental section.

Source of hepatic mitochondria	No. of experiments	Time (min)	Luciferase assay for total adenine nucleotides (nmol/mg of mitochondrial protein)				Isotope assay for [^{14}C]ATP taken up (nmol/mg of mitochondrial protein)			
		...	0	2	5	10	0	2	5	10
Foetal rats (21 day)	3		2.65 ± 0.07	4.12 ± 0.16	4.65 ± 0.15	5.39 ± 0.81	2.08 ± 0.25	4.02 ± 0.16	5.22 ± 0.29	6.39 ± 0.49
Foetal rats (21 day) + 10 nmol of potassium atractyloside	4		2.90 ± 0.22	3.91 ± 0.31	4.96 ± 0.51	5.02 ± 0.43	1.73 ± 0.05	3.74 ± 0.14	5.62 ± 0.54	6.44 ± 0.18
Suckling rats (3 day)	3		8.47 ± 0.52	9.31 ± 0.09	10.48 ± 0.12	12.71 ± 1.69	4.54 ± 0.54	8.52 ± 0.67	10.31 ± 0.92	12.59 ± 1.89
Suckling rats (3 day) + 10 nmol of potassium atractyloside	3		8.83 ± 0.47	9.06 ± 0.49	10.09 ± 0.57	10.78 ± 0.61	2.25 ± 0.15	8.10 ± 0.69	10.55 ± 0.73	11.74 ± 0.69
Adult rats	1		12.84	13.86	13.94	15.28	9.57	14.33	16.21	17.52
Adult rats + 10 nmol of potassium atractyloside	3		12.51 ± 0.48	14.39 ± 0.69	15.05 ± 1.10	17.36 ± 0.72	3.39 ± 0.40	12.17 ± 1.03	14.52 ± 0.76	16.03 ± 0.67

translocase activity was assayed at 0°C (Sandor & Pollak, 1976). Hence equilibration of labelled and non-labelled ATP can be expected not to be rate-limiting in foetal mitochondria (Table 1). Since there is good agreement between the two methods for all experimental times with foetal rat liver mitochondria and after 5 and 10 min incubation with suckling and adult rat liver mitochondria the more convenient isotope method was used for all subsequent experiments.

Mechanism of the adenine nucleotide accumulation

From the total adenine nucleotide determinations by the luciferase method it became also apparent that, at least at zero time, there is virtually no non-specific binding of ATP, as the zero-time values shown in Table 1 agree well with previously published values for the adenine nucleotide content of liver mitochondria isolated from rats of different developmental ages (Sutton & Pollak, 1978). The total adenine nucleotide content of 1 day pre-term rat liver mitochondria was 2.62 nmol/mg of protein, that of 3 day suckling rat liver mitochondria was 9.36 nmol/mg of protein and that of adult rat liver mitochondria was found to contain 12.70 nmol/mg of protein (Sutton & Pollak, 1978).

It should be pointed out here that mitochondria from glucagon-injected adult and foetal rats accumulate adenine nucleotides *in vivo* (Prpic *et al.*, 1978; Sutton & Pollak, 1980). In the present study the accumulation of adenine nucleotides occurs to a much greater extent in foetal than in suckling or adult rat liver mitochondria, particularly when the accumulation of adenine nucleotides is considered in terms of percentage increases. In Table 2 the rates of ATP accumulation of mitochondria obtained from rats of six different developmental ages are compared. The results indicate that foetal rat liver mitochondria increase their adenine nucleotide concentration by about a factor of four over a period of 10 min when the adenine nucleotide uptake is measured with the isotope method in the presence of atractylate. Hence all these preliminary experiments show clearly that after incubation with 1 mM-ATP and after subsequent isolation and washing, the mitochondria were enriched in their adenine nucleotide content. However these experiments do not indicate if the adenine nucleotide uptake is an active transport process; the increased ATP content could also be due to diffusion, or due to protein binding on the surface or within the matrix compartment. A number of different approaches are presented that attempt to show that ATP is transported across the inner mitochondrial membrane by a carrier-facilitated transport mechanism.

The adenine nucleotide content of foetal rat liver mitochondria has been shown to be less than 3 nmol/mg of protein (Nakazawa *et al.*, 1973; Sutton & Pollak, 1978; Table 1). After 10 min incubation with 1 mM-ATP and subsequent isolation and washing of these mitochondria, their adenine nucleotide content is increased by about 4 nmol/mg of protein to 7 nmol/mg protein (Tables 1 & 2). For the purpose of calculating mitochondrial adenine nucleotide concentrations it may be assumed that most of the 7 nmol are contained within the boundaries of the mitochondrial inner membrane. This sucrose-inaccessible space is generally thought to have a volume of 0.4–0.8 μl/mg of protein (Harris & van Dam, 1968). Assuming even a volume of 1 μl/mg of mitochondrial protein, this would mean that the initial concentration of adenine nucleotides within the mitochondrion is 2–3 mM, the concentration in the medium is 1 mM and the final concentration in the mitochondrion is 7 mM. Therefore the accumulation of ATP by foetal rat liver mitochondria represents an uptake against a concentration gradient. It should be noted here that the ATP concentration of the incubation medium (1 mM), is comparable with the ATP concentration present in foetal liver, which is of the order of 1.5 mM (Sutton & Pollak, 1978).

Experiments were carried out that indicated that

Table 2. *Mitochondrial ATP accumulation at different developmental ages*

These data represent mean values ± S.E.M. for ATP uptake as measured by the isotope method. All mitochondria were preincubated with 10 nmol of atractyloside for 1 min. The incubation, the inhibitor-stop with mersalyl and the processing of the incubation mixture were all carried out as described in the Experimental section.

Source of hepatic mitochondria	No. of experiments	Time (min) ...	ATP accumulation (nmol/mg of mitochondrial protein)			
			0	2	5	10
Foetal rats (17 day)	4		1.56 ± 0.07	4.19 ± 0.04	5.55 ± 0.33	6.96 ± 0.32
Foetal rats (19 day)	4		1.59 ± 0.19	3.24 ± 0.56	4.55 ± 0.51	5.51 ± 0.42
Foetal rats (21 day)	9		1.79 ± 0.13	3.48 ± 0.14	6.00 ± 0.49	7.29 ± 0.54
Suckling rats (3 day)	6		2.36 ± 0.32	7.82 ± 0.36	9.98 ± 0.42	11.19 ± 0.55
Suckling rats (5 day)	3		3.99 ± 0.53	9.13 ± 1.28	13.50 ± 0.21	15.26 ± 0.24
Adult rats	5		3.22 ± 0.36	10.63 ± 1.39	13.15 ± 1.24	14.99 ± 0.97

most of the accumulated [^{14}C]ATP was retained on reincubation with fresh incubation medium in the absence of ATP. In these experiments it was also found that, on repeating the washing procedure up to three times with incubation medium, at least 90% of the ATP was retained by the mitochondria; after a second wash with 0.25 M-sucrose, 81% of the ATP taken up was retained, whereas a second wash with water removed 82% of the ATP (J. K. Pollak & R. Sutton, unpublished work). The fact that washing the mitochondria in ionic medium or in the 0.25 M-sucrose medium results in only marginal losses of [^{14}C]ATP, whereas washing in water removes most of the labelled ATP from the mitochondrial pellet, suggests that most of the ATP was present within the boundaries of the mitochondrial inner membrane.

The accumulation of ATP by foetal rat liver mitochondria may also be explained in terms of passive diffusion followed by binding to proteins within the confines of the mitochondrial inner membrane. However, the results presented in Table 3 suggest that the ATP taken up by the mitochondria is freely available at least to the adenine nucleotide translocase, and hence the concept of passive diffusion followed by tight protein binding has to be abandoned. The results presented in Table 3 show that over 3 nmol of non-labelled ATP are taken up by foetal mitochondria in 10 min. Subsequent incubation for 2 min at 0°C with [^{14}C]ATP results in the exchange-diffusion of most of the mitochondrial ATP by the adenine nucleotide translocase, indicating that during the incubation at 30°C sufficient non-labelled ATP is taken up into the mitochondrial matrix to serve as substrate for the adenine nucleotide translocase to catalyse the exchange-diffusion of 5.72 nmol of ATP/mg of protein. The results presented in Table 3 also show that, for 2 min at 0°C, no ATP uptake, but only exchange-diffusion, occurs.

The presence of free or available ATP was further confirmed by using a different approach. Mitochondria were lysed osmotically by suspension in water and freezing and thawing them, and the supernatant of the lysed mitochondria was collected by centrifugation. This supernatant, containing the soluble matrix content, was placed onto a Sephadex G-25 column that was eluted with 0.05 M-NaCl. Any protein-bound ATP should be associated with the protein peak; on the other hand, free ATP extracted from the matrix should elute more slowly. In Fig. 2 it is shown that, after osmotic lysis with water, only free [^{14}C]ATP was detected.

The difference in the rate of ATP and ADP uptake and the lack of any significant AMP accumulation (Fig. 1) also points to the presence of an active transport mechanism, since passive diffusion would favour AMP, which is the least charged compound of these three adenine nucleotides.

Specificity of the adenine nucleotide transporter

The specificity of the adenine nucleotide transporter was evaluated by preincubating foetal rat liver mitochondria with related compounds and observing the inhibitory effect that these related nucleotides had on ATP transport (Table 4). Preincubation with non-labelled ADP, AMP, or dATP significantly inhibited the transport of [^{14}C]ATP, whereas cyclic AMP inhibited slightly, and P_i actually enhanced, this transport. p[NH]ppA has no inhibitory action, nor did GTP or CTP inhibit ATP uptake to any significant extent. The fact that p[NH]ppA did not inhibit ATP transport is of particular interest, since it had been shown that this analogue is able to substitute for ATP in increasing the respiratory control index of foetal rat liver mitochondria (Pollak, 1975). That action of ATP and p[NH]ppA was considered to occur on the outer surface of the mitochondrial inner membrane (Pollak, 1975, 1977), and since p[NH]ppA did not inhibit ATP transport,

Table 3. *Availability of accumulated ATP for the adenine nucleotide translocase*

Mitochondria from 21 day foetal rats were used. Incubation conditions were as described in the Experimental section. Both non-labelled and ^{14}C-labelled ATP were used as indicated. At the conclusion of the final incubation the mitochondria were processed as described in the Experimental section for the luciferase method and portions were assayed by both the luciferase and the isotope method.

Incubation conditions	Luciferase assay of total adenine nucleotides (nmol/mg of mitochondrial protein)	Isotope assay of [^{14}C]ATP taken up (nmol/mg of mitochondrial protein)
ATP (1 μmol), 0 min, 30°C	3.05	—
ATP (1 μmol), 10 min, 30°C	6.25	—
[^{14}C]ATP (1 μmol), 2 min, 0°C	2.60	2.16
ATP (1 μmol), 0 min, 30°C, washed and followed by incubation with [^{14}C]ATP (1 μmol), 2 min, 0°C	2.85	2.12
ATP (1 μmol), 10 min, 30°C, washed and followed by incubation with [^{14}C]ATP (1 μmol), 2 min, 0°C	5.67	5.72

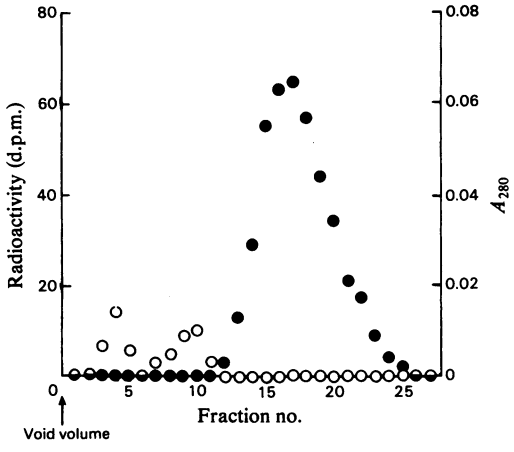

Fig. 2. *Distribution of accumulated [^{14}C]ATP as free [^{14}C]ATP and protein-bound [^{14}C]ATP*

Mitochondria from 21 day foetal rats were incubated with [^{14}C]ATP for 10min at 30°C in the presence of 10nmol of atractyloside as described in the Experimental section. At 10min the incubation was stopped with mersalyl and the mitochondria centrifuged and washed as described. The mitochondria were lysed by suspension in 1ml of water and freezing and thawing. The lysate was centrifuged in the Eppendorf centrifuge for 5min and a portion of the supernatant (0.5ml) was placed onto a Sephadex G-25 column (6ml) and eluted with 0.05M-NaCl. Fractions (five drops; 0.25ml) were collected in scintillation vials on 2.5cm GF/C Whatman glass filter disks and dried at 70°C. Scintillation fluid was added and the samples were counted for radioactivity as described in the Experimental section. Another portion (0.5ml) was similarly eluted through the same Sephadex G-25 column with 0.05M-NaCl. The fractions (five drops) were made up to 3ml and their absorption was measured at 260nm and 280nm in a Philips 8–1000 spectrophotometer. Only the absorption values at 280nm are shown, as the peaks at 260nm (though smaller) coincided with those at 280nm. ●, Radioactivity; O, A_{280}.

Table 4. *Effect of adenine nucleotides and other compounds on the accumulation of [^{14}C]ATP by foetal rat liver mitochondria*

Liver mitochondria isolated from 21 day foetal rats were incubated for 5min and [^{14}C]ATP accumulation was measured by the isotope method described in the Experimental section. The mitochondria were preincubated for 1min with the compounds listed in the Table. All experiments were carried out in the presence of 10nmol of atractyloside which was added for the preincubation period. The control value was obtained by measuring ATP uptake in the presence of actractyloside and 1μmol of [^{14}C]ATP, 100% being regarded as the difference of the ATP content (nmol/mg of mitochondrial protein) between the 0min and 5min incubations. The incubation with succinate was carried out in a stirred cell, open to the air and at the end of the incubation period the total contents were transferred to an Eppendorf tube for processing. Results are presented as means ± S.E.M.; the number of experiments is given in parentheses. Hexokinase (type III catalysing the conversion of 5.2μmol of glucose/min at pH 8.5) was from Sigma.

	[^{14}C]ATP accumulation	
Additions	% of control	% inhibition
None (control)	100	—
ADP (1μmol)	66 ± 2 (3)	34
AMP (1μmol)	67 ± 6 (3)	33
p[NH]ppA (1μmol)	100 ± 8 (3)	0
dATP (1μmol)	33 ± 9 (3)	67
Cyclic AMP (1μmol)	107 (1)	—
GTP (1μmol)	97 ± 7 (3)	3
CTP (1μmol)	99 ± 4 (3)	1
K_2HPO_4/KH_2PO_4 (1μmol)	169, 112	—
Mersalyl (0.05μmol)	23 ± 1 (3)	77
CCCP (0.001μmol)	47 ± 7 (3)	53
KCN (1μmol)	40, 60	50
Sodium succinate	74 (1)	26
Hexokinase (400μg) + glucose (6μmol)	33 (1)	67

the effect on the respiratory control should be considered to be unrelated to the accumulation of ATP. This is also borne out by the fact that the addition of ATP to foetal mitochondria during a respiratory control experiment will cause a marked increase in State-3 respiration within a few seconds (Pollak, 1975), although any significant uptake of ATP occurs only over a period of several minutes (Table 2).

Other compounds that inhibit ATP transport are mersalyl, which inhibits to the extent of 77%, CCCP, which inhibits by 55% and KCN, which inhibits to the extent of 52% (Table 4); oligomycin, rotenone or antimycin had no inhibitory action (J. K. Pollak & R. Sutton, unpublished work). The results presented in Table 4 therefore suggest the involvement of a thiol group or groups in the transport of ATP, as well as the requirement of a membrane potential, since an uncoupler such as CCCP considerably decreases the transport of ATP. Furthermore, the strong inhibition of ATP by mersalyl provided an opportunity to include mersalyl routinely as an inhibitor-stop at the end of the incubation period before commencing centrifugation; this procedure was used to increase the accuracy of the timed measurements of adenine nucleotide uptake.

Since mersalyl also inhibits the uptake of P_i it is of special interest that P_i does not inhibit ATP transport, suggesting that a separate carrier is involved.

The addition of succinate with oxygenation did not enhance the uptake of ATP (Table 4), showing that ATP uptake was not dependent on respiration; it was therefore unexpected that KCN (100 μM) did inhibit ATP uptake (Table 4).

Some kinetic parameters of the transporter

In preliminary experiments it had been established that the reaction rate, when expressed per mg of mitochondrial protein, was independent of the mitochondrial protein concentration between 0.25 and 2.0 mg/ml. In the experiments described here, the protein concentration was varied between 0.5 and 1.0 mg per incubation mixture of 1 ml.

The rate of ATP transport was dependent on the ATP concentration within the medium, as shown by a double-reciprocal plot (Fig. 3) which indicates that the K_m and V for the ATP transporter of 21 day foetal rat liver mitochondria are 2.2 mM and 4 μmol·min^{-1}·(mg of protein)$^{-1}$, respectively.

Two experiments were also carried out to measure the accumulation of [^{14}C]GTP by foetal rat liver mitochondria. The same methodology was used as for [^{14}C]ATP uptake experiments; it was found that within 2 min the GTP content increased from 0.8 to 1.6 nmol/mg of protein, but increased to only 1.94 nmol/mg of protein after 10 min. No attempt was made to characterize further the transport of this nucleotide.

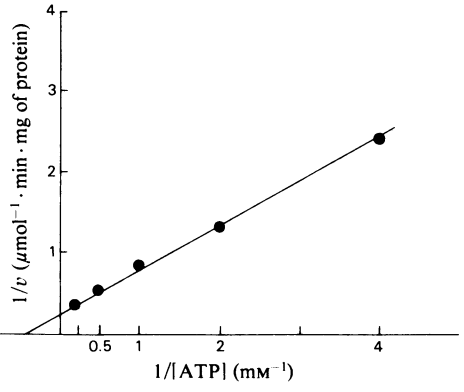

Fig. 3. *Lineweaver–Burk double reciprocal plot of [^{14}C]ATP uptake*

Mitochondria (0.59 mg of protein) isolated from 21 day foetal rat liver were incubated with different amounts of [^{14}C]ATP for 5 min at 30°C as described in the Experimental section (the rate of [^{14}C]ATP uptake was linear up to 5 min). Atractyloside was included during the preincubation period. The reaction was stopped, the mitochondria washed, processed and counted as described previously. The zero-time value for [^{14}C]ATP was deducted from the 5 min value and this value was used for plotting the curve. The K_m was 2.2 mM.

Low mitochondrial adenine nucleotide concentration in vivo and high ATP transport activity in vitro

In this preliminary study it has been established that foetal rat liver mitochondria possess a mechanism for the uptake of ATP. This therefore raises the more puzzling question of why foetal rat liver mitochondria have such a relatively low adenine nucleotide concentration (Sutton & Pollak, 1978) in the presence of such a carrier system. It may be speculated either that some inhibitor is present or that some essential factor for ATP transport is missing in foetal liver; this factor is either removed or supplied respectively after birth, or during isolation and incubation of the mitochondria *in vitro*.

It has been shown by Ureta *et al.* (1975) and subsequently by Hommes & Everts (1978), that in foetal rat liver more than 50% of the hepatocyte hexokinase activity appears to be bound to mitochondria; this binding of hexokinase by mitochondria decreases to very low levels within 20 days after birth (Ureta *et al.*, 1975). During this time the total hexokinase activity per g of liver also diminishes drastically (Ureta *et al.*, 1975). Both these groups of workers have suggested that the binding of hexokinase to mitochondria is not only a property of the changing hexokinase isoenzymes, but may also depend on some specific property of the mitochondrial membranes, which also change their characteristics during development (Ureta *et al.*, 1975; Hommes & Everts, 1978). This binding of hexokinase to the mitochondria and the greater amount of hexokinase present in foetal rat liver may be one of the factors contributing to the low adenine nucleotide concentration of foetal rat liver mitochondria.

This concept was put to the test by adding hexokinase and glucose to the incubation medium before the assay of ATP transport. In Table 4 it is shown that the addition of hexokinase does in fact inhibit ATP uptake by foetal rat liver mitochondria. The omission of Mg^{2+} from the incubation mixture lowered the ATP uptake to the same extent as did the addition of hexokinase (Tables 4 and 5). It has been demonstrated in a number of investigations that the presence of cations such as Mg^{2+}, K^+ or La^+ in low-ionic-strength media increases the rate of uptake of anions such as malate or P_i (Meisner *et al.*, 1972) and pyruvate (Papa *et al.*, 1971). Meisner *et al.* (1972) have also proposed that cations or protons, by binding to amphoteric groups on the outer surface of the mitochondrial inner membrane, increase the positive charge density, causing a

decrease of the apparent K_m of the anion that is to be transported across the membrane with no change in V. In view of these proposals it is suggested that in foetal rat liver the Mg^{2+} concentration available to the adenine nucleotide transporter may be rate limiting, either because Mg^{2+} is present only in low concentrations in foetal rat liver mitochondria or because most of it is bound to the hexokinase molecules that are associated with foetal rat liver mitochondria. Determination of the Mg^{2+} content by atomic absorption showed only small differences between foetal mitochondria on the one hand and mitochondria obtained from suckling or adult rats on the other hand. Foetal rat liver mitochondria contained 17nmol of Mg^{2+}/mg of protein, suckling rat liver mitochondria contained 27nmol of Mg^{2+}/mg of protein and adult rat liver mitochondria contained 25nmol of Mg^{2+}/mg of protein. (The determinations were kindly carried out by Dr. Julia James and Dr. R. Whittaker of the Chemistry Department, University of Sydney, using a Varian Techtron AA 6 atomic absorption spectrophotometer at 285.2nm, with a 0.5mm slit and a 3s integrated output and an air/acetylene gas mixture). This difference by itself may be considered to be too small to account for the inactivity of the ATP carrier, but it is proposed that a significant proportion of that Mg^{2+} is associated with the bound hexokinase; therefore the low adenine nucleotide concentration of foetal rat liver mitochondria could be explained in terms of the available Mg^{2+} concentration as suggested above.

Confirmatory evidence was obtained from experiments in which Mg^{2+} was either omitted or varied in the incubation medium (Table 5). The ATP uptake of 3-day suckling rat liver mitochondria was less affected by the absence of Mg^{2+} (J. K. Pollak, unpublished work). Hence the absorption of hexokinase and the non-availability of Mg^{2+} could be considered at this stage as a possible reason for the low adenine nucleotide content of foetal rat liver mitochondria. Furthermore it was found that the addition of Ca^{2+} to the incubation medium further enhanced ATP uptake (Table 5). This activation was significantly lower in the absence of Mg^{2+} (Table 5). ATP uptake by adult rat liver mitochondria (90nmol/20min per mg of protein) in the presence of 10mM-Mg^{2+} and 4mM-Ca^{2+} has been previously observed by Carafoli & Lehninger (1964), who also found that Mg^{2+} and Ca^{2+} were required for optimal ATP accumulation.

It has previously been suggested (Meisner, 1973), and it is relevant to this discussion, that a lack of Mg^{2+} may act as a rate-limiting factor for ATP-supported State-3 respiration, resulting in a low respiratory control index. In a recent investigation evidence has been provided showing that the low respiratory control index of the tightly coupled foetal rat liver mitochondria is the result of a limitation imposed on State-3 respiration (Sutton & Pollak, 1980). This limitation may be overcome by foetal rat liver mitochondria *in vitro* by adding ATP to a medium containing Mg^{2+} (Hallman, 1971; Nakazawa *et al.*, 1973; Pollak, 1975), or *in vivo* by parturition (Pollak, 1975). In addition it has been shown by Hallman (1971) that in foetal rat liver mitochondria the uncoupler-stimulated ATPase activity has an absolute requirement for Mg^{2+}. This dependence is lost shortly after birth (Hallman, 1971), further confirming the key position that Mg^{2+} holds during the perinatal period.

The adenine nucleotide transport system of foetal rat liver mitochondria has been shown to be atractylate-insensitive and mersalyl-sensitive. It may be possible to explain the increase in the mitochondrial adenine nucleotide concentration that occurs in the newborn rat in terms of changes in the Mg^{2+} and Ca^{2+} concentrations that are triggered at birth. Furthermore it should be pointed out again that the adenine nucleotide transporter is of fundamental importance for the maintainence of mitochondrial integrity in general and plays its part in mitochondrial biogenesis.

Table 5. *Effect of Mg^{2+} and Ca^{2+} on the ATP uptake by foetal rat liver mitochondria*

Mitochondria isolated from 21 day foetal rat liver were incubated with 1μmol of [^{14}C]ATP for 5min at 30°C and subsequently were processed as described in the Experimental section. The $MgCl_2$ concentration was varied and $CaCl_2$ was added as indicated. The ATP uptake (5min −0min) at 5mM-$MgCl_2$ was regarded as the 100% (control) value. The changes in ATP uptake due to changes in $MgCl_2$ and $CaCl_2$ concentration are expressed as a percentage of the control value ± S.E.M. whenever applicable. Individual results are given in parentheses.

$[MgCl_2]$ (mM)	$[CaCl_2]$ (μM)	No. of experiments	ATP uptake (% of control value)
5	—	5	100
—	—	5	35 ± 7
0.2	—	1	46
1.0	—	2	57 (43, 71)
10	—	2	86 (81, 91)
—	50	3	47 ± 4
—	100	3	49 ± 7
—	200	3	70 ± 13
—	500	2	180 (187, 173)
5	20	2	97 (91, 103)
5	50	4	130 ± 13
5	100	3	162 ± 27
5	200	4	246 ± 43
5	500	2	296 (261, 330)

Note Added in Proof (Received 5 August 1980)

Since submitting this paper for publication a relevant paper on adenine nucleotide transport into mitochondria has come to our notice [Aprille, J. R. & Asimakis, G. K. (1980) *Arch. Biochem. Biophys.* **201**, 564–575].

We thank Miss Wendy Mooney for expert technical assistance. This research was supported by a University of Sydney research grant and also by a grant from the Australian Research Grants Committee.

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