Biochem. J. (1976) **160**, 383–393
Printed in Great Britain

Location of an Oligomycin-Insensitive and Magnesium Ion-Stimulated Adenosine Triphosphatase in Rat Spleen Mitochondria

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(*Received* 19 *July* 1976)

1. When rat spleen mitochondria are incubated with oxidizable substrates, added $MgCl_2$ (>150 μM free concentration) markedly stimulates state-4 respiration and lowers both the respiratory control and ADP/O ratios; this effect is reversible on addition of excess of EDTA. 2. With [γ-^{32}P]ATP as substrate, an Mg^{2+}-stimulated ATPase (adenosine triphosphatase) was identified in the atractyloside-insensitive and EDTA-accessible space of intact rat spleen mitochondria. 3. Oligomycin has no effect on the activity of the Mg^{2+}-stimulated ATPase at a concentration (2.0 μg/mg of protein) that completely inhibits the atractyloside-sensitive reaction. Of the two ATPase activities, only the atractyloside-sensitive reaction is stimulated (approx. 40%) by dinitrophenol. 4. On digitonin fractionation the atractyloside-insensitive Mg^{2+}-stimulated ATPase co-purifies with the outer-membrane fraction of rat spleen mitochondria, whereas (as expected) the atractyloside-sensitive activity co-purifies with the inner-membrane plus matrix fraction. 5. Stoicheiometric amounts of ADP and P_i are produced as the end products of ATP hydrolysis by purified outer-membrane fragments; no significant AMP production is detected during the time-course of the reaction. 6. The outer-membrane ATPase is present in rat kidney cortex and heart mitochondria as well as in spleen, but is absent from rat liver, thymus, brain, lung, diaphragm and skeletal muscle.

Mitochondria isolated from rat spleen differ in several respects from those isolated from rat liver. The yield per g of fresh tissue is much lower owing to the smaller number of mitochondria per cell (van Bekkum, 1956), although, morphologically, the individual mitochondria are smaller and less uniform in size than those isolated from rat liver. One of the more striking differences is the apparent inertness of spleen mitochondria to agents like KCl, Ca^{2+} and *p*-chloromercuribenzoate, which cause immediate and extensive swelling in rat liver mitochondria (Arcos *et al.*, 1967). The sensitivity of lymphoid tissues to X-irradiation damage has also led to the observation that cytochrome *c* is much less tightly bound to the inner membranes of spleen and thymus mitochondria than it is in the liver and is consequently more easily released, leading to a lesion in oxidative phosphorylation that results in rapid death of the X-irradiated cells (Scaife, 1966).

When spleen mitochondria are isolated in an isoosmotic sucrose medium, suitable for liver, they respond to substrate plus ADP addition in a 'loosely coupled' manner so that rapid state-3 respiration does not cease when the theoretical quantity of added ADP is exhausted; in this respect they resemble guinea-pig heart mitochondria (Cleland & Slater, 1953; Chance & Baltschefsky, 1958; Chao & Davis, 1972). However, when they are isolated in the same medium containing EGTA,* as described for rat kidney-cortex mitochondria (Gmaj *et al.*, 1974), and incubated in a medium free of added bivalent metal ions, spleen mitochondria are 'tightly coupled' and give high respiratory control and ADP/O ratios. A similar effect of added bivalent metal ions has been observed with rat cerebral-cortex mitochondria (Sugano & Nagai, 1971).

In intact spleen mitochondria, the total ATPase activity observed in the absence of added Mg^{2+} is completely blocked by 50 μM-atractyloside. This basal activity is sensitive to oligomycin and is dependent on the retention of endogenous Mg^{2+} within the intact matrix space. When exogenous Mg^{2+} is added there is a marked stimulation of an additional ATPase activity that is not suppressed by either atractyloside or oligomycin. Ca^{2+} also stimulates the activity of this enzyme, a phenomenon that has been noted previously with rat heart sarcosomal preparations (Packer, 1958). These results are consistent with the view that there is a bivalent-metal-ion-stimulated ATPase activity in rat spleen mitochondria that continuously regenerates ADP at a site 'external' to the adenine nucleotide translocase.

The existence of several ATPases in whole mitochondria or submitochondrial particles has been

* Abbreviations: EGTA, ethanedioxybis(ethylamine)-tetra-acetic acid; ATPase, adenosine triphosphatase.

suggested by several workers on the basis of indirect evidence such as differential sensitivity to inhibitors (Hemker, 1963). In rat liver mitochondria Ohnishi & Ohnishi (1962) have extracted an ATPase resembling actomyosin, which appears to be different from the Mg^{2+}-requiring and 2,4-dinitrophenol-stimulated ATPase that has been semi-purified by Pullman *et al.* (1960). Further, in rat liver mitochondria, Beyer (1960) has observed the release of at least two different ATPases following prolonged ultrasonic treatment.

At present there is no conclusive evidence for or against the presence of more than one type of ATPase in mammalian mitochondria. In the present paper we describe two different ATPases from rat spleen mitochondria that can be clearly distinguished on the basis of their sensitivity to inhibitors, their requirement for added bivalent metal ions and their location within the mitochondria. A preliminary account of this work has already appeared (Vijayakumar & Weidemann, 1976).

Experimental

Materials

$[^{32}P]P_i$ was from the Australian Atomic Energy Commission, Lucas Heights, N.S.W., Australia. Digitonin was from Sigma Chemical Co., St. Louis, MO, U.S.A. It was recrystallized from hot ethanol, dried and ground to a fine powder. A stock solution 2%, (w/v) was prepared by adding warm 0.25M-sucrose to the powdered digitonin; it was then cooled to 0°C before use. Oligomycin (15% oligomycin A and 85% oligomycin B) was from Sigma. Atractyloside was a gift from Professor E. M. Klingenberg of the Institute for Physical Biochemistry, University of Munich, W. Germany. $CaCl_2$ of the highest purity available was from Orion Research Inc., MA, U.S.A. All other chemicals used were of analytical grade.

Methods

Isolation of mitochondria. Male albino Wistar rats (7 weeks old) were stunned by a blow on the head and exsanguinated. The spleens were removed quickly and placed in an ice-cold isolation medium containing sucrose (0.25M), EGTA (1.0mM), bovine serum albumin (1%, w/v) and Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl) ethanesulphonic acid] (5.0mM; pH7.4). The pH of the medium was adjusted to pH7.4 with KOH before use. The pooled spleens (approx. 5g of tissue) were minced coarsely with scissors and suspended in 9vol. of isolation medium. The mince was homogenized with two passes of a motor-driven Potter–Elvehjem tissue grinder. The homogenate was centrifuged at 650*g* for 10min and the pellet was discarded. The supernatant was centrifuged at 8500*g* for 15min, and the resulting mitochondrial pellet was washed twice with a wash medium (isolation medium free of EGTA) and centrifuged between washes at 8500*g* to resediment the pellet. The mitochondrial pellet was finally resuspended in a suitable volume of wash medium to give a final protein concentration of 20mg/ml.

Thymus tissue mitochondria were isolated under identical conditions. Mitochondria from rat skeletal muscle were isolated in iso-osmotic KCl medium by the method of Azzone & Carafoli (1960); and mitochondria from other rat tissues were isolated in iso-osmotic sucrose medium by the following methods: liver and kidney (Johnson & Lardy, 1967); heart (Cleland & Slater, 1953); brain (Ozawa *et al.*, 1966); lung (Fisher *et al.*, 1973).

Measurement of mitochondrial respiration. Respiration was measured with a Clark-type oxygen electrode (Reed, 1972). The reaction mixture consisted of sucrose (0.25M), EDTA or EGTA (1.0mM), potassium phosphate buffer (10.0mM; pH7.4), bovine serum albumin (1.0%, w/v), Hepes (10.0mM; pH7.4), 0.5–0.8mg of mitochondrial protein and, where applicable, $MgCl_2$ (0.9mM, giving a calculated concentration of free Mg^{2+} ions of approx. 150μM) or $CaCl_2$ (0.6mM giving a calculated concentration of free Ca^{2+} ions of approx. 50nM) in a final volume of 2.0ml. Free concentrations of the bivalent metal ions were determined on the basis of published chelator–metal-ion stability constants (Sillén & Martell, 1964) by using the computation method described by Perrin & Sayce (1967). Mitochondrial protein was determined by the biuret method of Layne (1957). The terminology of Chance & Williams (1956) for various mitochondrial states and for respiratory control ratios is used.

Digitonin fractionation of mitochondria. Rat spleen mitochondria were incubated with different concentrations of recrystallized digitonin (0.5–2.5mg per 10mg of mitochondrial protein) for 20min at 0°C. At the end of the incubation period the mitochondrial solutions were diluted with 3vol. of iso-osmotic sucrose and differentially centrifuged to prepare various mitochondrial fractions as described by Schnaitman & Greenawalt (1968) for rat liver mitochondria. Assay of specific marker enzymes (by methods given below) indicated that the inner and outer membranes of spleen mitochondria separated distinctly at 2.0mg of digitonin/10mg of mitochondrial protein.

Enzyme assays. Monoamine oxidase (marker enzyme for mitochondrial outer membrane) was assayed by the method of Tabor *et al.* (1954); cytochrome oxidase (marker enzyme for mitochondrial inner membrane) by the polarographic method of Schnaitman *et al.* (1967); glutamate dehydrogenase (marker enzyme for mitochondrial matrix) by the method of Schmidt (1974); adenylate kinase (EC 2.7.4.3) (marker enzyme for mitochondrial inter-

membrane space) by the method of Bergmeyer *et al.* (1974). Creatine kinase was assayed essentially by the method of Forster *et al.* (1974). ATP pyrophosphohydrolase (EC 3.6.1.8) activity was assayed by the method of Lieberman *et al.* (1967). Inorganic pyrophosphatase was assayed by using the method of Lowry (1957) except that the P_i liberated was assayed as described by Dulley (1975). 5'-Nucleotidase was assayed essentially as described by Ray (1970), except that liberation of P_i was used as a measure of the activity of the enzyme.

Analytical methods. ATP was assayed by the method of Lamprecht & Trautschold (1974); AMP and ADP were assayed by the method of Jaworek *et al.* (1974). P_i was assayed by the modified method of Fiske & SubbaRow (1925) as described by Dulley (1975).

Synthesis of [γ-^{32}P]ATP. [γ-^{32}P]ATP was synthesized by a modification of the method of Glynn & Chappell (1964) as described by Post & Sen (1967).

Assay of ATPase activity. (*a*) With intact mitochondria. The reaction medium contained sucrose (0.25M), EGTA or EDTA (1.0mM), Hepes (25mM; pH7.4), $MgCl_2$ (0.9mM, giving a calculated concentration of free Mg^{2+} ions of approx. 150μM), unlabelled ATP (150μM) and [γ-^{32}P]ATP (3×10^6 d.p.m.) in a final volume of 3.0ml. The reaction was started by the addition of mitochondria (0.2–0.5mg of protein).

(*b*) With the isolated outer-membrane preparation. The reaction medium used was identical with (*a*) except that sucrose was replaced by water. The reaction was started by addition of mitochondrial outer membrane preparation (40–60μg of protein).

In each case samples (200μl) were withdrawn from the assay mixture after different time-intervals (shown in the legends) and were placed in ice-cold $HClO_4$ (20μl, 1.0M). The mixture was centrifuged (2min at 5000*g*) in an Eppendorf centrifuge to sediment the precipitated protein, and a sample (100μl) of clear supernatant was neutralized with KOH (10μl; 1.0M). The labelled P_i liberated was separated from unhydrolysed [γ-^{32}P]ATP chromatographically (Zweig & Sherma, 1972), by using Whatman no. 1 paper. The paper strips were cut into six equal pieces (3.0cm) and placed in vials containing 10ml of scintillation fluid {15g of butyl-PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole], dissolved in 1.5litres of toluene and 1.0litre of 2-methoxyethanol}. The ^{32}P radioactivity was determined in a Beckman LS-350 liquid-scintillation counter. The P_i liberated was calculated from the ^{32}P peak radioactivity.

Results and Discussion

Effect of Mg^{2+} and Ca^{2+} on spleen mitochondrial oxidation

Fig. 1(*a*) shows that spleen mitochondria incubated in the absence of added bivalent metal ions were tightly coupled, giving an ADP/O ratio of 2.9 and a respiratory control ratio of not less than 8.0 for pyruvate. The addition of free Mg^{2+} (>150μM) prolonged the state-3 respiration rate (Fig. 1*b*), which returned to state 4 only after the addition of excess of EDTA (600μM). EDTA also restored the cyclic response to added ADP. In contrast, the addition of free Ca^{2+} (50nM) to spleen mitochondria oxidizing pyruvate (Fig. 1*c*) blocked the initiation of state-3 respiration on addition of ADP in a manner that was not reversible by EDTA, presumably due to the formation of a Ca–NADH complex in the matrix space (Chance, 1965; Vinogradov *et al.*, 1972). Free Ca^{2+} at this concentration also inhibited the oxidation of other NAD-linked substrates tested (e.g. β-hydroxybutyrate, glutamate and citrate). Succinate was oxidized at a normal rate under these conditions, but with a very low respiratory control ratio (1.1) that can be attributed to Ca^{2+}-induced uncoupling (Rossi & Lehninger, 1964).

Fig. 2 shows the effect of increasing the concentrations of free Mg^{2+} and free Ca^{2+} on the 'tightness' of coupling of rat spleen mitochondria oxidizing pyruvate plus malate. At low concentrations (<20μM), free Mg^{2+} increased the respiratory control and ADP/O ratios (Fig. 2*a*) by causing a small increase in the state-3 respiration and decreasing that of state 4. When the free Mg^{2+} concentrations exceeded 20μM, the respiratory control and ADP/O ratios both decreased, as at this concentration there was a significant stimulation of the state-4 rate. The state-4 rate continued to increase in response to added Mg^{2+} until the mitochondria were apparently completely 'uncoupled' at free Mg^{2+} concentrations in excess of 150μM. Very low free Ca^{2+} concentrations had an effect identical with that of low free-Mg^{2+} concentration on spleen mitochondrial oxidation (Fig. 2*b*), but at concentrations in excess of 40nM the respiratory control and the ADP/O ratios decreased dramatically. With pyruvate as substrate, owing to Ca–NADH complex formation (Vinogradov *et al.*, 1972), there was no further stimulation of the state-4 rate at higher Ca^{2+} concentrations. Addition of succinate, however, stimulated both the state-3 and state-4 rates under these conditions, but the ADP/O and the respiratory control ratios remained low and unaltered.

Differentiation between matrix (oligomycin-sensitive) and external (oligomycin-insensitive) ATPase activities from spleen mitochondria

The results of the polarographic studies described above are consistent with the presence of a bivalent-metal-ion-dependent ATPase-like activity in intact rat spleen mitochondria that is not present in liver. The release of [^{32}P]P_i from [γ-^{32}P]ATP was used, therefore, as a more specific assay for the activity of this enzyme. Fig. 3 shows that, even without added

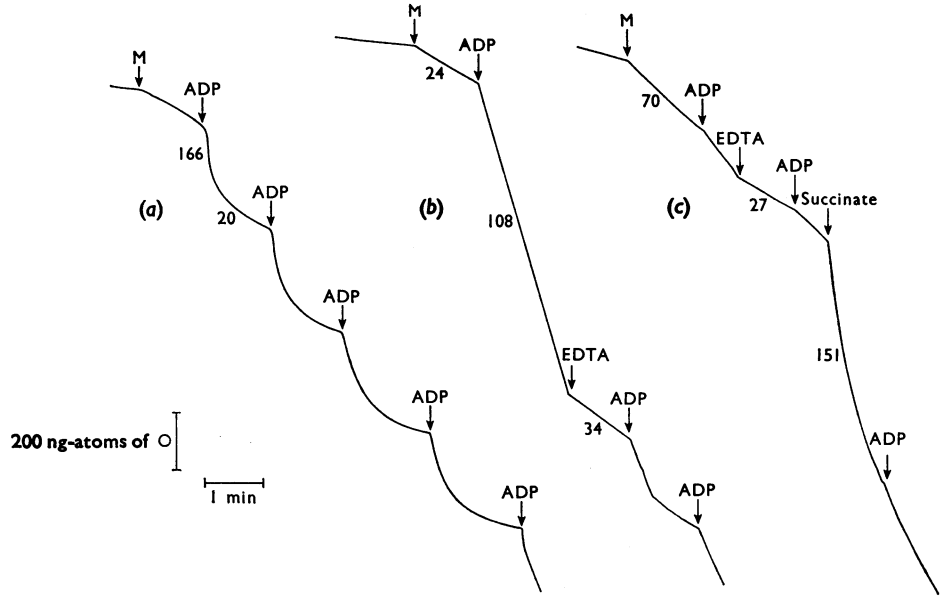

Fig. 1. *Influence of Ca^{2+} and Mg^{2+} on O_2 consumption of rat spleen mitochondria*

The results are presented as oxygen-electrode traces showing the effect of free Mg^{2+} (150 μM) and free Ca^{2+} (50 nM) on spleen mitochondria oxidizing pyruvate (3.0 mM) plus malate (0.5 mM). The mitochondria (M) (1.0 mg) were suspended in a total reaction volume of 2.0 ml, as given in the Experimental section, and the respiration was stimulated by adding ADP (200 nmol). EDTA (600 μM) and succinate (1.0 mM) were added as indicated by arrows. The values under the oxygen-electrode traces are the rates of O_2 consumption in nmol/min per mg of protein. (*a*) Control, containing no added bivalent metal ions; (*b*) plus total $MgCl_2$ (0.9 mM); (*c*) plus total $CaCl_2$ (0.6 mM).

Mg^{2+}, the spleen mitochondria possessed an ATPase activity that was completely suppressed by either atractyloside (50 μM) or oligomycin (2 μg/mg). This activity represents the matrix ATPase that is independent of added Mg^{2+}. When free Mg^{2+} (150 μM) was added to the incubation medium in the presence of 50 μM-atractyloside, there was a marked stimulation of the rate of $[^{32}P]P_i$ liberation. This additional ATPase activity, which is accessible to [γ-^{32}P]ATP confined to the outside of the atractyloside barrier, can be differentiated from the matrix ATPase, as it is insensitive to oligomycin (in the range 2–10 μg/mg) and dependent on added bivalent metal ions for its activity.

Effect of uncoupler on spleen mitochondrial ATPase activities

With tightly coupled spleen mitochondria in the absence of added Mg^{2+}, the matrix-space ATPase was stimulated by about 40% on addition of 0.1 mM-dinitrophenol (Table 1). This rate represents the maximum oligomycin-sensitive ATPase activity (148 nmol/min per mg of protein) observed in the present experiments, and it should be noted that its specific activity is approx. equal to that of the Mg^{2+}-stimulated ATPase (168 nmol/min per mg of protein). In contrast, the Mg^{2+}-stimulated ATPase activity measured in the presence of oligomycin (10 μg/mg) was not significantly accelerated by dinitrophenol addition. The small stimulation observed (8–10%) is probably due to slight mitochondrial damage sustained during preparation, with the concomitant release of a fraction of the oligomycin-sensitive ATPase activity from the matrix.

Location of the oligomycin-insensitive ATPase of spleen mitochondria

Inner and outer membranes of rat spleen mitochondria were separated by digitonin treatment and differential centrifugation, by using the specific marker enzymes described for rat liver mitochondria (Schnaitman & Greenawalt, 1968). As the digitonin concentration was increased (Fig. 4*a*) the activity of monoamine oxidase, the specific marker enzyme for mitochondrial outer membranes, also increased in the high-speed pellet and reached a maximum at

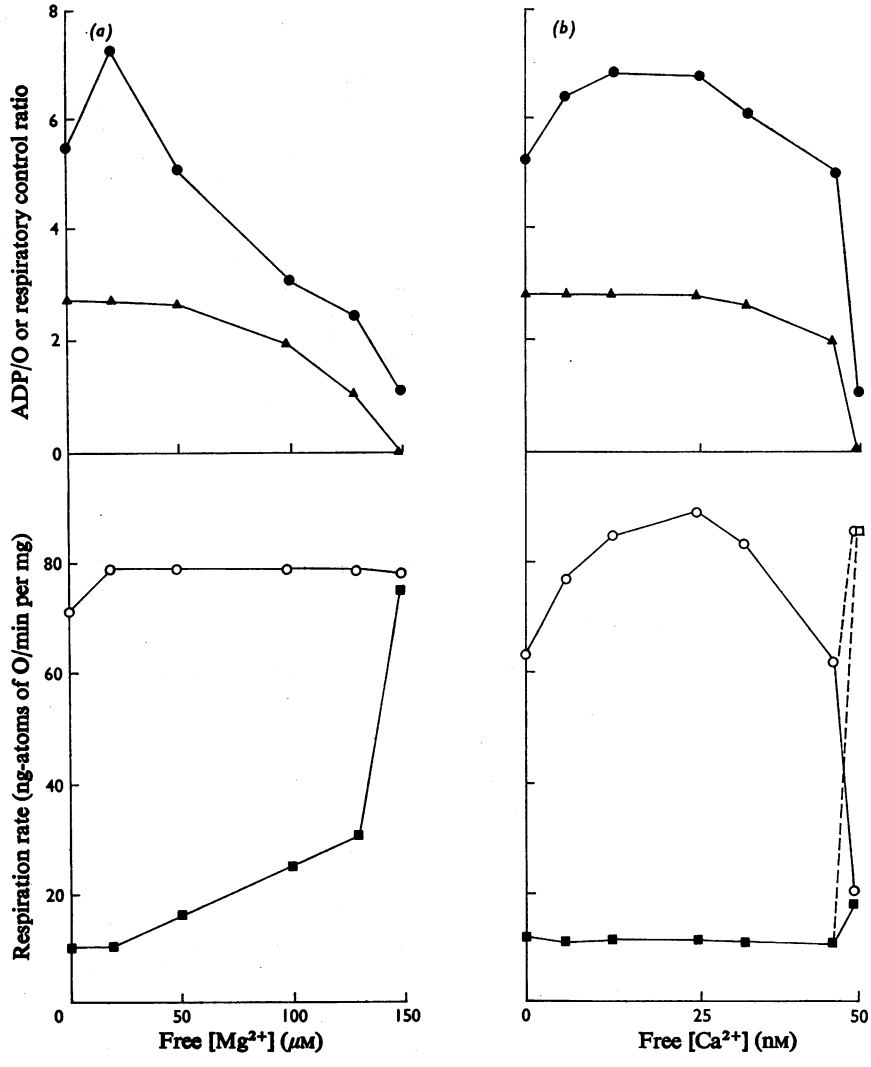

Fig. 2. *Effect of Mg^{2+} (a) and Ca^{2+} (b) on ADP/O and respiratory control ratios*

Rat spleen mitochondria (1.0mg) were added to 2.0ml of reaction mixture containing pyruvate (3.0mM) plus malate (0.5mM) and various free concentrations of $MgCl_2$ and $CaCl_2$ as indicated (calculated from total concentrations as given in the Experimental section). The respiration was then stimulated by adding ADP (200nmol). Succinate (1.0mM) was added, as indicated by the broken lines, when free Ca^{2+} concentrations greater than 40nm were used in the incubation medium. The respiratory control ratio (●), ADP/O ratio (▲), state-3 (○) and state-4 respiration rates (■ for pyruvate plus malate and □ for succinate) are plotted separately for each condition.

2.0mg of digitonin/10mg of mitochondrial protein. Over the same digitonin concentration range adenylate kinase, the mitochondrial periplasmic-space marker, was released into the high-speed supernatant fraction (Fig. 4*b*) and disappeared from the low-speed pellet (Fig. 4*c*), which contained the major portion of the cytochrome *c* oxidase and glutamate dehydrogenase activities. At concentrations below 2mg of digitonin the marker enzymes of the inner-membrane plus matrix space fractions were barely detectable in the high-speed pellet and supernatant fractions (Figs. 4*a* and 4*b*), which is additional evidence for the intactness of the mitoplast fraction. When the concentration of digitonin was increased to more than 2.0mg/10mg of protein, the marker enzymes were distributed non-specifically in all fractions.

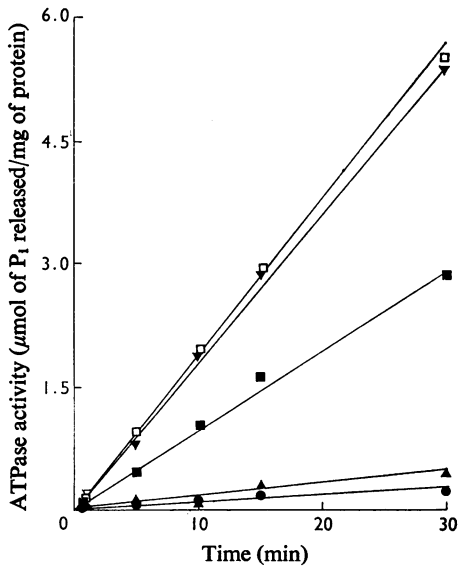

Fig. 3. *Differentiation between oligomycin-sensitive and -insensitive ATPases of rat spleen mitochondria*

ATPase activities were assayed by using intact mitochondria (0.4mg) in the incubation medium containing [γ-^{32}P]-ATP (specific radioactivity 10^6d.p.m./μmol) as described in the Experimental section, under the following conditions: ■, mitochondria alone; ▲, plus atractyloside (50μM); ●, plus oligomycin (2μg/mg); ▼, plus $MgCl_2$ (0.9mM) and atractyloside (50μM); □, plus $MgCl_2$ (0.9mM) and oligomycin (2μg/mg).

Table 1. *Effect of dinitrophenol on rat spleen mitochondrial ATPase activities*

The ATPase activity was assayed in intact mitochondria (0.4mg), at 22°C as described in the Experimental section. [γ-^{32}P]ATP (specific radioactivity 3.5×10^6d.p.m./μmol) was added to give a final concentration of 150μM in each case in a total assay volume of 3.0ml.

Additions	Activity (nmol of P_i/min per mg)
Intact mitochondria	108
Oligomycin (10μg/mg)	12
Dinitrophenol (0.1mM)	148
$MgCl_2$ (0.9mM)+oligomycin (10μg/mg)	180
$MgCl_2$+oligomycin (10μg/mg) +dinitrophenol (0.1mM)	192

The specific activities of marker enzymes were analysed in the submitochondrial fractions obtained at 2.0mg of digitonin/10mg of mitochondrial protein (Table 2). More than 80% of the total mitochondrial protein remained in the low-speed pellet, which also contained increased specific activities of the marker enzymes of the inner-membrane and matrix space fractions, including the oligomycin-sensitive ATPase. Only 7–8% of the total mitochondrial protein was recovered in the high-speed pellet; in this fraction a selective 9–12-fold enhancement of the specific activities of monoamine oxidase and oligomycin-insensitive ATPase indicated that both of these activities co-purified with the outer-membrane subfraction. Approx. 11–13% of the total mitochondrial protein was released into the inter-membrane soluble-space fraction, which contained adenylate kinase and creatine kinase of greatly enhanced specific activity (7-fold).

Of the various fractions obtained by digitonin treatment, approx. 90% of the total oligomycin-insensitive ATPase activity was recovered with the outer membranes. The small amount of activity (8%) still associated with the inner membranes is a result, possibly, of incomplete separation of the two membranes. Taken together, these results indicate that the oligomycin-insensitive Mg^{2+}-stimulated ATPase of spleen mitochondria is located predominantly in the outer membrane, although (as expected) the oligomycin-sensitive ATPase is located almost exclusively in the inner membrane plus matrix space.

Stoicheiometry of the ATPase reaction

Fig. 5 shows that the products of the Mg^{2+}-stimulated ATPase reaction catalysed by the washed outer-membrane fragments were ADP and P_i, produced in equimolar amounts, exactly equal to the loss of ATP from the medium. No significant production of AMP was detected during the time-course of the reaction, which is consistent with the absence of adenylate kinase and ATP pyrophosphohydrolase activities from this fraction after washing (Table 3).

Examination of the outer-membrane fraction for contamination with other ATPase activities

Outer-membrane preparations were tested for possible plasma-membrane contamination, with (Na^++K^+)-dependent ATPase as the marker enzyme, by assaying the Mg^{2+}-stimulated ATPase activity in the presence of ouabain (0.01–0.1mM). Ouabain had no significant effect on the Mg^{2+}-stimulated activity when tested over this concentration range, indicating the absence of plasma-membrane contamination. This conclusion was further confirmed by the total absence of 5′-nucleotidase activity from intact spleen mitochondria and from all of the mitochondrial subfractions (Table 3).

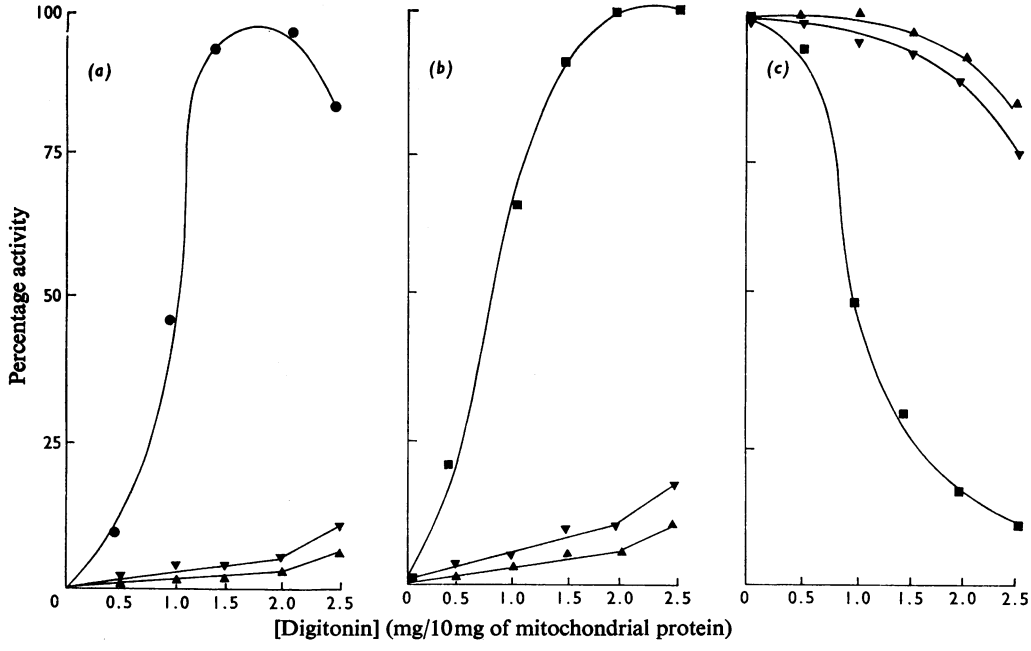

Fig. 4. *Differential fractionation of rat spleen mitochondria with digitonin*

Spleen mitochondria were treated with different concentrations of digitonin (0.5–2.5mg/10mg of protein), and various mitochondrial fractions [outer-membrane fraction (Fig. 4*a*), intermembrane soluble fraction (Fig. 4*b*) and inner-membrane plus matrix fraction (Fig. 4*c*)] were prepared by differential centrifugation as described in the Experimental section. Specific marker enzymes of different fractions (●, monoamine oxidase; ▲, cytochrome oxidase; ▼, glutamate dehydrogenase; ■, adenylate kinase) were assayed as described in the text.

Of other possible contaminating activities, only 2% of the total creatine kinase activity, measured in the direction of ATP synthesis from ADP and creatine phosphate, was present in the unwashed outer-membrane fraction. A similar proportion of the inorganic pyrophosphatase activity, which also co-purified with adenylate kinase and creatine kinase from the periplasmic space, was found in this subfraction. Table 3 shows that all of these contaminating activities disappeared from the outer-membrane preparations after three washes.

Tissue distribution

Well-coupled mitochondria from various tissues were prepared by standard methods and their respiration was studied in the presence of succinate in an incubation medium free from added bivalent metal ions; the effect of adding Mg^{2+} or Ca^{2+} was tested once state-4 respiration had been established. Table 4 shows that Mg^{2+} (0.9mM) stimulated the state-4 respiration in only kidney-cortex, brain, heart and spleen mitochondria, whereas Ca^{2+} (0.6mM) invariably stimulated the rate in all mitochondrial preparations owing to its uptake at the expense of ATP hydrolysis. In subsequent experiments, where the ATPase activity was assayed by following the specific release of $[^{32}P]P_i$ from [γ-^{32}P]ATP in the presence of atractyloside, the results show clearly that only spleen, kidney-cortex and heart mitochondria possessed this distinctive ATPase activity. The highest specific activity was observed with heart mitochondria, which exceeded that of spleen by at least sixfold. Brain mitochondria did not release $[^{32}P]P_i$ under these conditions, so in this case the Mg^{2+}-stimulated state-4 rate observed is more likely to be due to the stimulation of a phosphotransferase activity like creatine kinase (Sugano & Nagai, 1971).

Nature of the ADP-regenerating reaction in Mg^{2+}-treated spleen mitochondria

The apparent loss of phosphorylation efficiency (ADP/O ratio) and the fall in acceptor control ratio observed in response to added Mg^{2+} with rat spleen mitochondria is similar to that reported for heart

Table 2. *Distribution of specific marker enzymes in digitonin-treated rat spleen mitochondria*

Rat spleen mitochondria were treated with 2.0mg of digitonin/10mg of protein and the various fractions were prepared as described in the Experimental section. Mg^{2+}-stimulated ATPase activity was measured in each fraction in the presence of 10μg of oligomycin/mg of protein; oligomycin was omitted in the assay for oligomycin-sensitive ATPase. [γ-^{32}P]ATP (specific radioactivity 3.0×10^6 d.p.m./μmol) was added to the assay medium as described in Table 1. The other enzymes listed were assayed as described in the text. Specific activities of all enzymes are expressed in nmol/min per mg of protein. The numbers of experiments are given in parentheses and the results are mean activities ± S.E.M.

		Specific activity (nmol/min per mg of protein)							
		Glutamate dehydrogenase (2)		Cytochrome *c* oxidase (2)		ATPase (oligomycin-sensitive) (3)		Adenylate kinase (3)	
Fraction	Protein (%) (5)	Specific activity	Total activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity	Total activity
Whole mitochondria	100	421	100	1071	100	108±11	100	287±11	100
Outer membranes (high-speed pellet)	7.2±1.7	1.1	0.2	86	5.7	14.2	9.4	14.2	5.2
Inner membranes+matrix (low-speed pellet)	81.2±6.9	592	114	1480	89.5	147	111	46	13.7
Intermembrane soluble space (high-speed supernatant)	13.9±2.3	4.7	1.5	129	15.6	0	0	2030	104
Recovery	102±4	—	116	—	111	—	120	—	118

	Creatine kinase (3)		Monoamine oxidase (3)		Mg^{2+}-stimulated ATPase (oligomycin-insensitive) (8)	
	Specific activity	Total activity	Specific activity	Total activity	Specific activity	Total activity
Whole mitochondria	76.5±6.1	100	12.1±2.4	100	178±7	100
Outer membranes (high-speed pellet)	1.3±0.2	1.7	147±17	87.5	2320±52	89.3
Inner membranes+matrix (low-speed pellet)	2.98±0.5	3.9	2.9±0.4	19.4	16.9±3.2	8.1
Intermembrane soluble space (high-speed supernatant)	561.9±37	102.7	—	—	0	0
Recovery	—	108	—	107	—	97.4

sarcosomes (Packer, 1957, 1958; Chao & Davis, 1972) and rat kidney-cortex mitochondria (Gmaj *et al.*, 1974). In the present work, mitochondrial damage was excluded as the basis for the Mg^{2+} stimulation of an ADP-regenerating reaction on the following grounds: (*a*) high respiratory control and ADP/O ratios were obtained with the same mitochondrial preparations in the absence of added Mg^{2+} (Fig. 1); (*b*) the Mg^{2+}-independent ATPase activity was suppressed completely by atractyloside (Fig. 3); (*c*) the atractyloside-insensitive ATPase activity, which required added Mg^{2+} for activation, was quite insensitive to oligomycin (Fig. 3), which argues against it being a contaminating ATPase activity released from the mitochondrial matrix; (*d*) the possibility that the addition of Mg^{2+} itself might cause an irreversible loss of mitochondrial integrity, and a concomitant stimulation of state-4 respiration, was ruled out by the restoration of both normal state-4 respiration and the cyclic response of the mitochondria to the addition of ADP on subsequent addition of excess of EDTA (Fig. 1). In addition, the differential fractionation of spleen mitochondria with digitonin (Table 3) has shown clearly that the outer-membrane fraction has more than 90% of the oligomycin-insensitive ATPase activity associated with it, which argues for a distinctive activity, not associated with mitochondrial damage, that is peculiar to spleen, heart and kidney-cortex mitochondria. Its presence and activity is not associated with 'aging' of the mitochondria, as there was neither an increase nor decrease in the Mg^{2+}-stimulated ATPase activity measured in the presence of oligomycin (10μg/mg of protein), atractyloside (50μm) and ouabain (0.1 mM) over a 5h period after

isolation of the mitochondria (E. K. Vijayakumar & M. J. Weidemann, unpublished work).

A number of other possible contaminating activities have been considered, and excluded, as the basis for an Mg^{2+}-stimulated ATPase reaction situated predominantly in the outer-membrane fraction. (The small percentage of this activity found in association with the inner membranes is considered to be due to incomplete separation of the two membranes.) The combined activities of ATP pyrophosphohydrolase and adenylate kinase, for example, could result in a 'pseudo-ATPase' activity and produce ADP by the reaction sequence shown in Scheme 1. Two findings argue against this being the reaction mechanism: (i) the fact that stoicheiometric amounts of ADP and P_i were produced as the only end products of the reaction catalysed by the outer-membrane fraction (Fig. 5); (ii) the virtual absence of ATP pyrophosphohydrolase, adenylate kinase and pyrophosphatase activities from the outer-membrane fraction after three washes (Table 3). Stimulation of creatine kinase activity by added Mg^{2+} could also result in a continuous regeneration of ADP from ATP with an apparent uncoupling effect, as has been reported for rat cerebral-cortex mitochondria (Sugano & Nagai, 1971), but this activity is completely absent from the washed outer-membrane fraction of spleen mitochondria. The outer-membrane fraction, in fact, was virtually free of all matrix, inner-membrane and intermembrane-space marker enzymes and was selectively enriched in monoamine oxidase as well as the oligomycin-insensitive ATPase activity.

The $(Na^+ + K^+)$-dependent ATPase from the plasma membrane, which may have partially sedimented with the microsomal fraction, was not a contaminant of the outer-membrane fraction. These

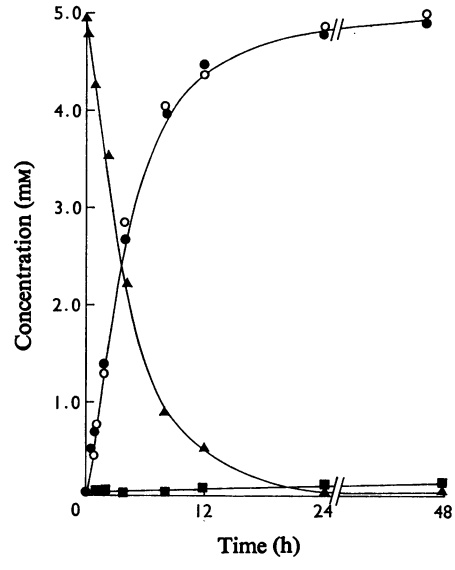

Fig. 5. *Stoicheiometry of the outer-membrane ATPase reaction from rat spleen mitochondria*

Hydrolysis of unlabelled ATP by the washed mitochondrial outer-membrane (200μg) ATPase was performed at 37°C in a sterile medium containing tetracycline (2mg/ml) as well as Hepes buffer (20.0mM, pH7.4), ATP (5.0mM) and $MgCl_2$ (8.0mM) in a final volume of 5.0ml. Samples (200μl) were withdrawn at different time-intervals, as indicated, and the reaction was stopped with ice-cold $HClO_4$ (20μl, 1.0M). All glassware was autoclaved at 120°C for 30min before use. Adenine nucleotides (▲, ATP; ●, ADP; ■, AMP) and P_i (○) were analysed as described in the text.

Table 3. *Effect of washing the outer-membrane preparation from rat spleen mitochondria on the disappearance of contaminating enzyme activities*

Mitochondrial outer membranes were prepared as described in the Experimental section and were washed between spins with Hepes buffer (20.0mM; pH7.4) before sedimentation at 100000*g* for 60min. The enzymes listed in the Table were assayed as described in the text. All values are expressed as percentages of the activity measured with intact mitochondria (given as 100%); specific activity is expressed as nmol/min per mg of protein. The numbers of observations are given in parentheses and the results are expressed as mean activities ± S.E.M.

Preparation assayed	Adenylate kinase (3)	Creatine kinase (3)	Inorganic pyrophosphatase (2)	ATP pyrophospho-hydrolase (2)	5'-Nucleotidase (2)
Specific activity in intact mitochondria	287 ± 11.1	76.5 ± 6.1	317.6	0	0
Percentage activity					
Intact mitochondria	100	100	100	—	—
Outer membranes					
Unwashed	5.2	1.7	2.3	0	0
After three washes	0	0	0.04	0	0

Table 4. *Tissue distribution of the outer-membrane ATPase activity*

Mitochondria from different rat tissues were isolated as described in the text. The mitochondrial respiration was measured in an incubation medium containing 1mM-succinate and either (i) no added bivalent metal ions or (ii) $MgCl_2$ (0.9mM) or (iii) $CaCl_2$ (0.6mM). The ATPase activity was assayed in an incubation medium containing [γ-^{32}P]ATP (0.15mM at a specific activity of 1.5×10^6 d.p.m./μmol), atractyloside (50μM) and either $MgCl_2$ (0.9mM) or $CaCl_2$ (0.6mM) in a final volume of 3.0ml.

Tissue (mitochondria)	State-4 respiration (ng-atoms of O/min per mg)			ATPase activity (nmol of P_i/min per mg)	
	No bivalent metal ions	$MgCl_2$ (0.9mM)	$CaCl_2$ (0.6mM)	$MgCl_2$ (0.9mM)	$CaCl_2$ (0.6mM)
Liver	21	28	98	12	4
Spleen	25	108	151	150	65
Thymus	32	41	115	14	7
Heart	30	142	158	760	442
Brain	28	104	114	17	14
Kidney cortex	31	89	97	71	47
Lung	23	59	120	12	19
Hind-leg muscle	29	31	117	12	27
Diaphragm	34	37	106	9	29

$$ATP + H_2O \xrightarrow{\text{ATP pyrophosphohydrolase}} AMP + PP_i \quad (a)$$

$$AMP + ATP \xrightarrow{\text{Adenylate kinase}} 2ADP \quad (b)$$

Scheme 1.

findings indicate that the oligomycin-insensitive ATPase of rat spleen is a distinctive mitochondrial enzyme present in the outer-membrane fraction, which is similar to the enzyme reported in kidney-cortex mitochondria by Gmaj *et al.* (1974).

We acknowledge the award of an Australian National University Research Scholarship to E. K. V. in connexion with this project. The work was supported by an Australian Research Grants Committee grant no. D 2 70/17436 to M. J. W.

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