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# Net Adenine Nucleotide Transport in Rat Kidney Mitochondria

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**This study investigated the hypothesis that changes in the adenine nucleotide (ATP + ADP + AMP) content of kidney mitochondria can occur by a transport mechanism that catalyzes net transfer of adenine nucleotides across the inner mitochondrial membrane. The adenine nucleotide content of isolated kidney mitochondria was 8.23 ± 0.85 nmol/mg mitochondrial protein. This amount increased or decreased as a function of the external [ATP–Mg] when mitochondria were incubated in phosphate-containing medium. The increases and decreases were inhibited to different extents by 100 μM EGTA (ethylene glycol bis(β-aminoethyl ether)*N,N'*-tetraacetic acid) or 5 μM carboxyatractyloside (CAT), suggesting two transport mechanisms. The unidirectional components (influx and efflux) of net flux were examined separately for the CAT-insensitive (EGTA-sensitive) and CAT-sensitive (EGTA-insensitive) mechanisms. CAT-insensitive adenine nucleotide influx and efflux were stimulated by $[Ca^{2+}]_{free}$ up to 2 μM; for ATP influx, $K_m$ was 1.7 mM, $V_{max}$ was 3.5 nmol/min/mg protein, and $Mg^{2+}$ was required. Efflux varied as a function of both the external and matrix [ATP] and was completely inhibited by mersalyl. ATP was a better substrate than ADP, and ADP transport did not require $Mg^{2+}$. The CAT-sensitive mechanism was characterized by studying phosphate-induced adenine nucleotide efflux. Efflux varied with external [$P_i$] and with matrix [ATP] and was not inhibited by cyclosporin. The amount of CAT required for maximal inhibition was 800 pmol/mg protein. In contrast to CAT-insensitive efflux, this pathway was only partially inhibited by mersalyl and showed no preference for ATP vs ADP. In conclusion, two distinct mechanisms for net adenine nucleotide transport were demonstrated. Both exchange adenine nucleotides (ATP–Mg or ADP) for $P_i$. One mechanism is identical to the CAT-insensitive ATP–Mg/$P_i$ carrier known in liver mitochondria; the other is a CAT-sensitive mechanism that is not present in liver and may represent a novel function of the ADP/ATP translocase or another CAT-sensitive carrier.** 

The adenine nucleotide (ATP + ADP + AMP) content of rat kidney mitochondria decreases *in vivo* under circumstances such as hypoxia and ischemia. It already has been shown that an ischemia-induced decrease in the mitochondrial adenine nucleotide content impairs respiration in kidney mitochondria. Upon organ reperfusion, or upon incubation of the isolated mitochondria with ATP, the matrix adenine nucleotide content and oxidative phosphorylation rates are restored (1, 2). In rat liver, the mitochondrial adenine nucleotide content also decreases reversibly under hypoxia and ischemia; there is an increase in a variety of other conditions such as glucagon or vasopressin treatment and during the transition from fetal to neonatal life (reviewed in 3). The mitochondrial adenine nucleotide content of heart mitochondria is also sensitive to ischemia and reperfusion (4).

Mechanisms which may account for changes in the mitochondrial adenine nucleotide content have been discussed (5). Some loss of adenine nucleotides during ischemia may be due to purine catabolism. However, changes in the mitochondrial adenine nucleotide content can sometimes occur while the total adenine nucleotide content of the tissue remains constant, and therefore must be brought about by net transfer of adenine nucleotides between the matrix and the cytoplasm. Any mitochondrial transport mechanism that allows net uptake and net loss of adenine nucleotides has to be different from the one-for-one exchange that is characteristic of the ADP/ATP translocase.

In rat liver mitochondria the well-characterized ATP–Mg/$P_i$ carrier accounts for net changes in the matrix adenine nucleotide content (3). In isolated heart mitochondria, net adenine nucleotide movements are observed but the transport mechanism is different from that in the liver and not as well understood (4). Net adenine nucleotide uptake has been demonstrated for ischemic kidney mitochondria, suggesting that a specific net transport

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process exists (2). The purpose of this study was to examine the transport mechanism in kidney mitochondria with regard to kinetic properties and regulation and to compare the results with the transport mechanisms in liver and heart. In order to do this we investigated both net uptake and loss and the unidirectional components (influx and efflux) of net adenine nucleotide transport in normal kidney mitochondria.

The results show that net adenine nucleotide movements occur as exchange for phosphate by two distinct pathways. One pathway is carboxyatractyloside-insensitive, and the mechanism, regulation, and kinetics appear identical to those of the ATP-Mg/$P_i$ carrier in liver mitochondria. The other pathway is carboxyatractyloside-sensitive and may involve a unique function or isoform of the ADP/ATP translocase.

## MATERIALS AND METHODS

*Preparation of mitochondria.* Mitochondria were isolated by differential centrifugation from the kidneys of fed adult male CD-1 rats, weighing 200–350 g (Charles River Laboratories). The renal cortex was separated from the medulla, homogenized in an ice-cold isolation medium of 250 mM sucrose, 0.1 mM EDTA, and 1 mM Tris-HCl (pH 7.4), and centrifuged at 600*g* for 10 min. The supernatant was centrifuged at 8000*g* for 10 min to collect mitochondria. The mitochondrial pellet was washed twice in the isolation medium. For the second washing step EDTA was omitted. The washed mitochondria were resuspended in sucrose–Tris to 25–30 mg protein/ml. Although mannitol–sucrose–Tris medium is often used to prepare kidney mitochondria, we used the sucrose–Tris medium because significantly higher respiratory control ratios and rates of uncoupled respiration were obtained when using glutamate + malate, which was the substrate in all our assays. Typically, respiration rates (measured polarographically in units of nmol $\frac{1}{2}$ $O_2$/min/mg mitochondrial protein) were 192 ± 33 for state 3 and always ≤10 for state 4, so that the respiratory control ratios were always >15 and usually around 20. Liver mitochondria were isolated in 0.25 M sucrose, 1 mM Tris, and 0.1 mM EDTA as previously described (6). Protein determinations were made by the Lowry *et al.* procedure (7).

*Incubation conditions.* Incubations of kidney mitochondria were carried out at 30°C in medium containing 225 mM sucrose, 2 mM $K_2HPO_4/KH_2PO_4$, 5 mM $MgCl_2$, 10 mM KCl, 10 mM Tris-HCl, 5 mM glutamate, 5 mM malate, pH 7.4, as described previously for liver mitochondria (6). Mitochondria were added so that the final concentration was approximately 1 mg protein/ml. ATP was present at concentrations indicated in the figures and tables. The ATP-Mg/$P_i$ carrier is $Ca^{2+}$ sensitive, so for some experiments $[Ca^{2+}]_{free}$ was controlled using Ca-EGTA[2] buffers (8). The incubation medium was the same except that 5 mM EGTA was included with a variable amount of $CaCl_2$. The total $[Ca^{2+}]$ needed to produce the desired $[Ca^{2+}]_{free}$ was calculated using the stability constants for Ca-EGTA, Mg-EGTA, Ca-ATP, and Mg-ATP from Bartfai (9). The concentrations of free $Ca^{2+}$ were measured with Indo-1 and calculated as $[Ca^{2+}]_{free} = K_d\,(F - F_{min})/(F_{max} - F)$ (10, 11). The buffer used in the $Ca^{2+}$ measurements was the same as that used in all transport assays except that $Mg^{2+}$ was present at a free concentration of 1 mM. Magnesium binds to Indo-1 with an unknown dissociation constant and interferes with the $Ca^{2+}$-induced fluorescence of the dye. The $K_d$ of 250 nM that we used to calculate the $[Ca^{2+}]_{free}$ concentrations in our measurements was determined with the $[Mg^{2+}]_{free}$ at 1 mM (11). The measured $[Ca^{2+}]_{free}$ values were corrected for a medium containing 5 mM $[Mg^{2+}]_{total}$ using the stability constants mentioned above and were in good agreement with calculated concentrations.

*Net accumulation and net loss of mitochondrial adenine nucleotides.* To measure net changes in the adenine nucleotide content, mitochondria were added to the incubation medium (time = 0) containing ATP concentrations of 2.0, 1.0, or 0.15 mM ATP. After desired time intervals between 45 s and 15 min, 1.3-ml aliquots were stopped with EGTA (final concentration 100 μM). The samples were stored on ice until the last time point was stopped. We determined that no EGTA-insensitive (carboxyatractyloside-sensitive) net transport occurs on ice (data not shown). Nevertheless, after carboxyatractyloside-sensitive (EGTA-insensitive) net transport was discovered, 5 μM carboxyatractyloside was included in the stop buffer along with EGTA. Samples were centrifuged (8000*g*) at 4°C for 2 min and washed once with the sucrose–Tris–EDTA buffer. The final pellet was resuspended to a protein concentration of 3 to 4 mg/ml. Neutralized perchloric acid extracts were prepared as described previously (12) and adenine nucleotides (ATP + ADP + AMP) were determined enzymatically (13, 14).

*Unidirectional transport.* The unidirectional components (influx and efflux) of net transport were measured separately, essentially as described previously (6). The incubation medium contained 5 μM carboxyatractyloside to inhibit ADP/ATP translocase. Thirty seconds after the addition of mitochondria, 1.0 mM ATP was added (time = 0). For ATP influx, the ATP added was trace-labeled with [$^{32}$P]ATP (~1500 cpm/nmol). For ATP efflux, the matrix pool of adenine nucleotides was uniformly labeled by pretreating the isolated mitochondria at ice temperature for 45 min with 4 nCi of carrier-free [$^{14}$C]ADP/mg of mitochondrial protein (specific activity of ADP, 56.6 mCi/mmol) as previously described (6). The extramitochondrial ATP in the adenine nucleotide efflux assays was unlabeled. Initial rates were measured by sampling onto filters at 15, 45, and 75 s, as previously described except that 100 μM EGTA was added to the 0.15 mM NaCl washing medium.

*Manipulation of matrix adenine nucleotide content.* In order to test the effect of different matrix adenine nucleotide contents on transport rates, the mitochondria were prepared as described previously (6). Briefly, the mitochondria were incubated with 0.15–4.0 mM ATP under standard conditions for 15 min at 30°C in the absence of carboxyatractyloside, centrifuged at 8000*g* for 10 min, and washed once. The mitochondria were finally resuspended in sucrose–Tris medium. The final adenine nucleotide content could be varied between 3 and 22 nmol/mg mitochondrial protein, depending on the ATP concentration used in the incubation; these mitochondria were then used in the usual way for transport assays.

*Other methods.* When it was necessary to determine the matrix concentrations of ATP and ADP, mitochondria were rapidly separated from the incubation medium by centrifugation of 1-ml aliquots layered over silicone oil and 12% perchloric acid (6, 16). The acid extracts of the pellets were neutralized and assayed enzymatically for adenine nucleotides. When adenine nucleotides were included in the incubations, the amount measured in the pellet was corrected for any amount trapped in the [$^{14}$C]sucrose permeable space. To determine matrix volume, the incubation medium contained [$^{14}$C]sucrose (0.2 μCi/ml) and [$^{3}$H]$H_2O$ (1.0 μCi/ml). Aliquots were layered over silicone oil and perchloric acid as described above. The matrix volume was determined as the difference between total [$^{3}$H]$H_2O$ space and [$^{14}$C]sucrose permeable space in the mitochondrial pellet (16), and this value was used to calculate matrix adenine nucleotide concentrations.

## RESULTS

### *Net Transport of Adenine Nucleotides*

The adenine nucleotide content of isolated normal kidney mitochondria was 8.23 ± 0.85 nmol/mg mitochondrial protein ($n$ = 44). When the mitochondria were incubated with ATP under standard conditions a change in the total content occurred over time. Either net uptake or net loss

[2] Abbreviations used: Ap5A, $P^1$,$P^5$-di(adenosine-5')-pentaphosphate; Indo-1, 1-[2-amino-5-(6-carboxyindol-2-yl)phenoxy]-2-(2'-amino-5'-methylphenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid; EGTA, 1,2-di(2-aminoethoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid.

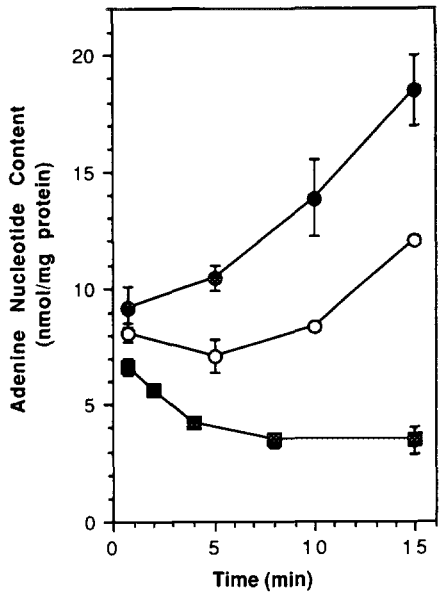


FIG. 1. Net transport of adenine nucleotides over time as a function of the external ATP concentration. Net transport was measured with external ATP concentrations of 2 mM (●), 1 mM (○), and 0.15 mM (■). Incubation conditions were as described under Materials and Methods. Data points are averages of several independent experiments.

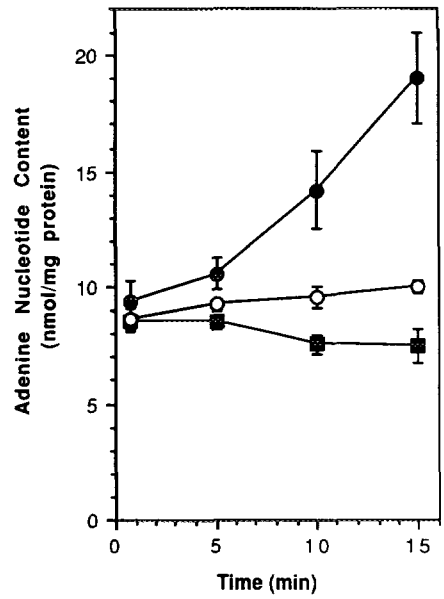


FIG. 2. Inhibition of net uptake of adenine nucleotides by mersalyl and EGTA. Net transport was measured with an external ATP concentration of 2 mM. Incubation conditions are described under Materials and Methods. A control (●) was compared to the presence of 200 μM mersalyl (○) or the presence of 100 μM EGTA (■) in the incubation medium. Data points are from several independent experiments.

was observed depending on the ATP concentration in the medium (Fig. 1). With 2 mM ATP the adenine nucleotide content of the mitochondria increased after 15 min to approximately twofold. With 1 mM ATP net uptake was less. With 0.15 mM ATP the mitochondria lost 50% of matrix adenine nucleotides and attained a steady state after about 8 min. With 2.0 and 1.0 mM ATP in the medium a steady state was not achieved even after 15 min.

As Fig. 2 shows, net uptake in the presence of 2 mM ATP was completely inhibited by either 200 μM mersalyl or 100 μM EGTA. Mersalyl also completely inhibited net loss in the presence of 0.15 mM ATP (Fig. 3) but with EGTA the initial rate of net loss of adenine nucleotides was reduced by only 55% (Fig. 4).

In further net transport experiments the effect of 5 μM carboxyatractyloside was tested. Figure 4 demonstrates that carboxyatractyloside alone reduced the initial rate of net loss by about 30%. However, with both EGTA and carboxyatractyloside present, net loss was almost completely inhibited. Carboxyatractyloside diminished the rate of net uptake with 2 mM ATP as well (Fig. 5). Figure 5 shows also that there is no net transport with carboxyatractyloside and 1 mM ATP in the incubation medium, compared to net uptake that was observed in the control in Fig. 1. This inhibition of ATP transport could have been secondary to a decrease in the ATP concentration in the mitochondrial matrix or in the medium, because carboxyatractyloside prevents the exchange of adenine nucleotides over the ADP/ATP translocase. Carboxyatractyloside did not increase the intramitochondrial ATP concentration (data not shown), presumably because glutamate + malate is available as a substrate for oxidative phosphorylation of matrix ADP. However, there was a 34% decrease in the ATP concentration in the medium over 15 min in the presence of carboxyatractyloside (Fig.

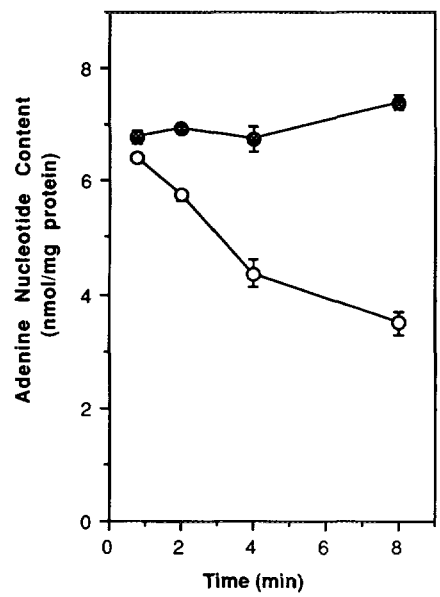


FIG. 3. Inhibition of net loss of adenine nucleotides by mersalyl. Net transport was measured with an external ATP concentration of 0.15 mM in the absence (○) and presence (●) of 200 μM mersalyl. Data points are averages of two experiments.

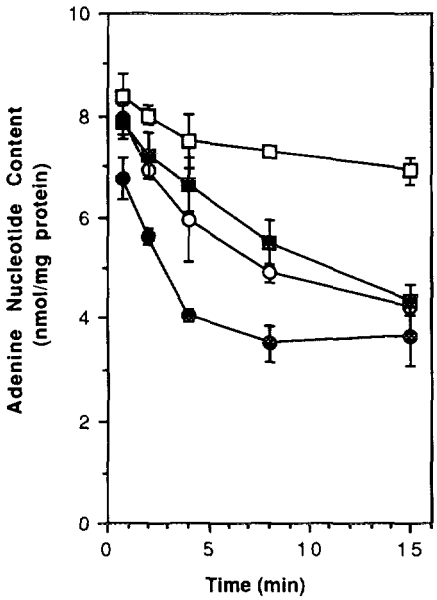


**FIG. 4.** Inhibition of net loss of adenine nucleotides by EGTA and carboxyatractyloside. Net transport was measured with an external ATP concentration of 0.15 mM. A control incubation (●) is compared to 5 μM carboxyatractyloside (○), to 100 μM EGTA (■), and to both 5 μM carboxyatractyloside and 100 μM EGTA (□). Data are averaged from several experiments.

6). With the addition of an effective ATP-regenerating system (creatinephosphokinase and phosphocreatine), the medium ATP concentration was maintained but the partial inhibition of net uptake by carboxyatractyloside remained unchanged (data not shown).

The decrease in the medium ATP concentration that occurred in the presence of carboxyatractyloside (Fig. 6) is likely to be the result of $F_1F_0$ ATPase activity in damaged mitochondria or of ATPases in other contaminating organelles such as endoplasmic reticulum. Carboxyatractyloside prevents the hydrolyzed external ATP from being rephosphorylated within the mitochondria by inhibiting the translocase. This decrease in medium ATP concentration is a linear function of incubation time, but after 2 min the ATP concentration falls only to 92% of the control. Hence when measuring initial rates of unidirectional transport between 0 and 75 s in the presence of carboxyatractyloside as shown below, the ATP concentration was not significantly affected even though an ATP-regenerating system was not used.

### *Characteristics of Carboxyatractyloside-Insensitive Unidirectional Influx and Efflux*

With 1.0 mM ATP (Fig. 5) there was initially no net uptake when carboxyatractyloside was present, indicating that influx and efflux rates must be approximately equal. Therefore, it was convenient to use 1 mM ATP as the standard concentration for influx and efflux assays. We first studied unidirectional fluxes in the presence of 5 μM carboxyatractyloside. Both unidirectional influx and efflux (carboxyatractyloside-insensitive) in the presence of 1 mM ATP were completely inhibited by 100 μM EGTA and 200 μM mersalyl, as was net uptake (Table I).

In the presence of 1.0 mM ATP and 5 μM carboxyatractyloside, unidirectional influx and efflux were examined with varied external $[Ca^{2+}]_{free}$. External $[Ca^{2+}]_{free}$ was controlled using Ca–EGTA buffers and calculated and measured as described under Materials and Methods. Figure 7 shows that unidirectional efflux and influx of adenine nucleotides are regulated by micromolar $[Ca^{2+}]_{free}$. A concentration of about 2.0 μM $[Ca^{2+}]_{free}$ was sufficient for maximal activation of adenine nucleotide movement in either direction. Without Ca–EGTA buffers, the rate of influx was 1.16 ± 0.14 nmol/min/mg protein, and efflux was 1.21 ± 0.23 nmol/min/mg protein. These rates were similar to maximal rates observed with ≥2 μM $[Ca^{2+}]_{free}$ added, which indicates that enough $[Ca^{2+}]_{free}$ is normally present in the medium to stimulate transport maximally. This nominal $Ca^{2+}$ is presumed to be a contaminant of the $MgCl_2$ reagent (8).

The rate of unidirectional influx, measured with [$^{32}$P]ATP in the presence of 5 μM carboxyatractyloside, was dependent on the external concentration of ATP (Fig. 8A). We studied influx with external ATP concentrations between 0.5 and 4.0 mM and obtained an apparent $K_m$ of 1.7 mM and a $V_{max}$ of 3.5 nmol/min/mg protein (Fig. 8B) under standard incubation conditions.

In order to study unidirectional efflux as a function of the total adenine nucleotide content in the mitochondria,

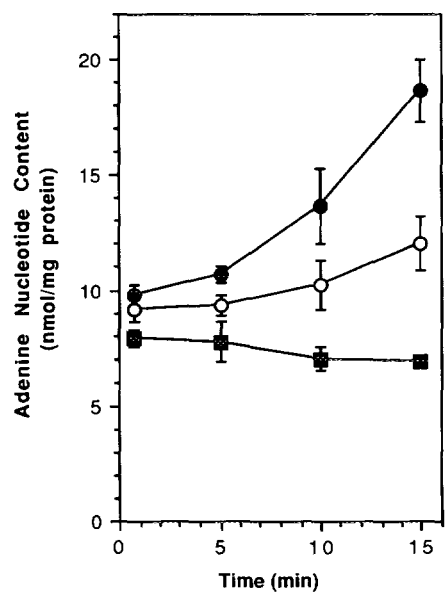


**FIG. 5.** Inhibition of net uptake of adenine nucleotides by carboxyatractyloside. Net transport was measured in the presence of external ATP in the absence and presence of 5 μM carboxyatractyloside. Symbols represent 1 mM ATP and carboxyatractyloside (■), 2 mM ATP control (●), and 2 mM ATP plus carboxyatractyloside (○). A control for 1 mM ATP may be found in Fig. 1.

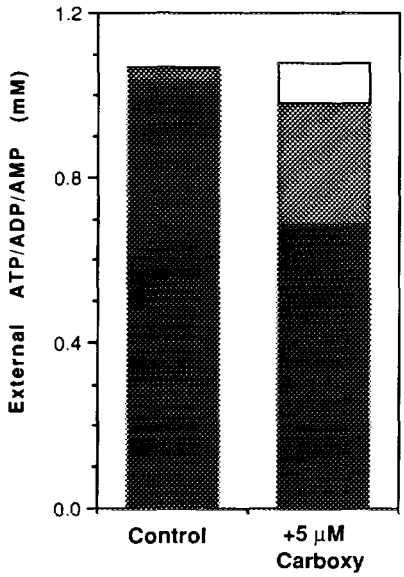


**FIG. 6.** Effect of carboxyatractyloside on adenine nucleotide concentrations in the external medium after incubation of kidney mitochondrial with 1 mM ATP. Mitochondria were incubated under standard conditions with 1 mM ATP in the absence (control) and presence of 5 μM carboxyatractyloside (carboxy). After incubation for 15 min, 1-ml aliquots of the assay were centrifuged over silicone oil as explained under Materials and Methods. Samples from the supernatant were acidified with PCA and then neutralized prior to the enzymatic determination of ATP (■), ADP (□), and AMP (□). Data are averages of four experiments. Results with 15 μM carboxyatractyloside (not shown) were the same as those shown here for 5 μM carboxyatractyloside.

TABLE I

Inhibition of Carboxyatractyloside-Insensitive Adenine Nucleotide Influx and Efflux by Mersalyl and EGTA

| | Influx | Efflux |
|---|---|---|
| | (nmol/min/mg protein) | |
| Control | 1.05 ± 0.01 | 1.24 ± 0.00 |
| 200 μM mersalyl | 0.17 ± 0.05 | 0.11 ± 0.02 |
| 100 μM EGTA | 0.07 ± 0.06 | 0.04 ± 0.03 |

*Note.* Unidirectional influx and efflux were measured under standard incubation conditions as described under Materials and Methods with 5 μM carboxyatractyloside added. Values are averages of two experiments.

the matrix adenine nucleotide content was manipulated as described under Materials and Methods. Efflux was measured with 1 mM ATP in the incubation medium. Results below suggest that ATP–Mg (rather than ADP) is the preferred substrate for carboxyatractyloside-insensitive transport of adenine nucleotides in either direction, and so we plotted efflux rates as a function of the matrix ATP concentration. Matrix ATP concentrations were determined under standard incubation conditions after 1 min as described under Materials and Methods. The matrix volume (0.87 μl/mg mitochondrial protein) did not vary as a function of the matrix adenine nucleotide content. In Fig. 9A, no saturation of efflux was observed in the concentration range examined. However, when evaluating efflux it is necessary to consider that the matrix phosphate concentration in energized mitochondria is severalfold higher than the standard phosphate concentration (2 mM) in the medium, which will increase (by competition) the apparent $K_m$ for ATP–Mg in the mitochondrial matrix. Predictably, the $K_m$ calculated from Fig. 9B for efflux was 8.6 mM, severalfold higher than for the influx $K_m$. A $V_{max}$ of 3.6 nmol/min/mg protein for ATP–Mg efflux was obtained with 1 mM ATP present in the medium, which is similar to the $V_{max}$ for influx under similar conditions. Furthermore, Fig. 9B shows that when efflux is induced by 2 mM $P_i$ alone (no ATP), both $K_m$ and $V_{max}$ are lower (2.1 mM and 0.9 nmol/min/mg protein, respectively).

Unidirectional efflux rates also varied as a function of the external ATP concentration (Fig. 10), which is further evidence that influx and efflux are tightly coupled. With no ATP in the medium the observed efflux is likely to be due to exchange of matrix adenine nucleotide for external phosphate. With a medium concentration of 4 mM ATP there is no additional stimulation of efflux compared to 2 mM ATP, because the available ATP and phosphate concentrations on the outside are high enough for a maximal stimulation of efflux. Under these circumstances, the rate-limiting factor for efflux is the matrix ATP concentration.

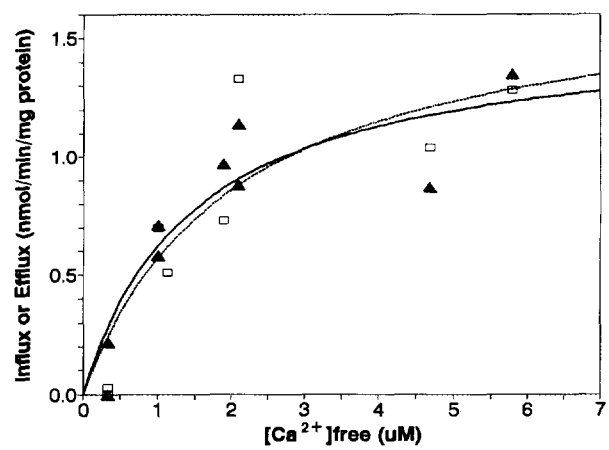


**FIG. 7.** Carboxyatractyloside-insensitive adenine nucleotide influx and efflux as a function of the free $Ca^{2+}$ concentration in the medium. Unidirectional influx and efflux were measured under standard conditions as described under Materials and Methods, with 5 μM carboxyatractyloside added. $[Ca^{2+}]_{free}$ was controlled with Ca–EGTA buffer and measured in separate assays fluorometrically with Indo-1 (see Materials and Methods). The initial rates of unidirectional influx (▲) and efflux (□) are shown as a function of the $[Ca^{2+}]_{free}$. Data points were collected from three separate experiments for influx, and from two for efflux. The curves were determined by nonlinear regression (21); the dotted line is efflux, the solid line is influx.

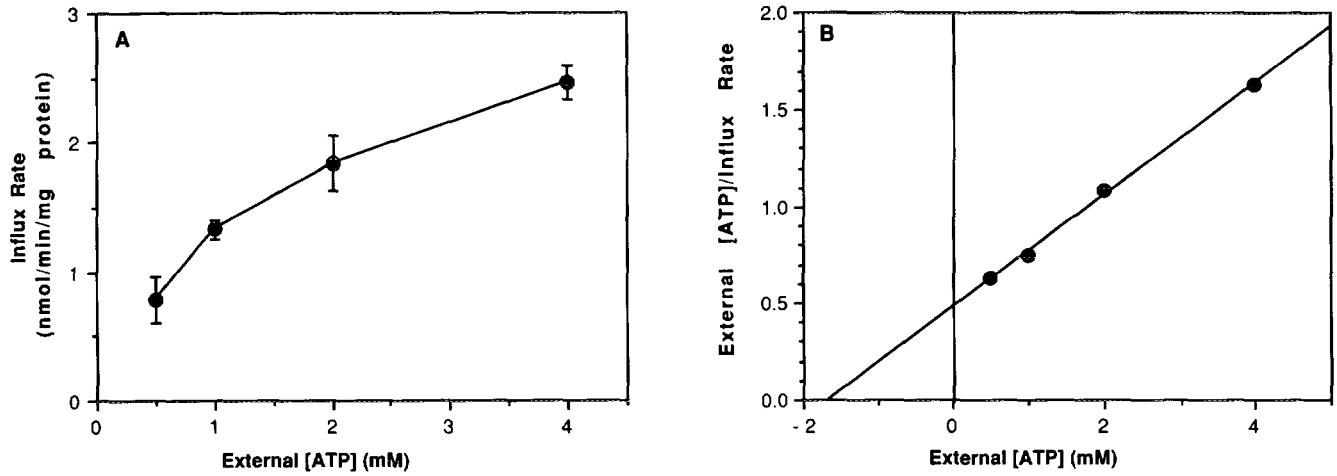


**FIG. 8.** Carboxyatractyloside-insensitive adenine nucleotide influx as a function of the external ATP concentration. Unidirectional influx of ATP (in the presence of 5 $\mu$M carboxyatractyloside) was measured with ATP between 0.5 and 4 mM. (A) Data are from three experiments. (B) [ATP]/influx against [ATP], which determines $K_m$ as 1.7 mM ($x$ intercept) and $V_{max}$ as 3.5 nmol/min/mg protein ($K_m/y$ intercept).

In order to determine the preferred substrate for efflux, we kept the adenine nucleotide content constant but varied the relative concentrations of matrix ATP and ADP by including 12 $\mu$g/ml oligomycin. Matrix ATP was thus reduced from 2.93 nmol/mg protein in the control to 0.97 nmol/mg protein with oligomycin, whereas the matrix ADP content was increased from 0.43 nmol/mg protein in the control to 3.17 nmol/mg protein with oligomycin. To calculate these values we subtracted any bound ATP or ADP determined as the amount remaining after the mitochondria were completely depleted of adenine nucleotides by exhaustive phosphate-induced efflux. In the experiment, efflux was determined with only 2 mM phosphate and no ATP in the medium to exchange for matrix adenine nucleotides. This enabled us to compare the results in Table II with those for the carboxyatractyloside-sensitive transport component described below. For the carboxyatractyloside-insensitive component of efflux in Table II, a rate of 0.66 nmol/min/mg was observed with a high matrix ATP/ADP ratio, compared to a rate of 0.35

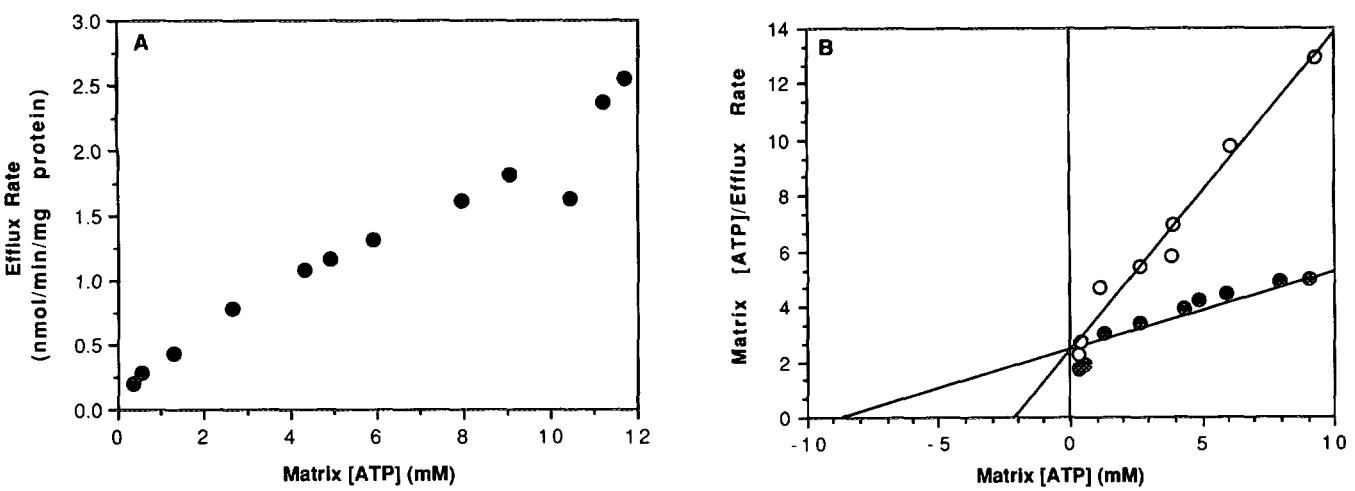


**FIG. 9.** Carboxyatractyloside-insensitive adenine nucleotide efflux as a function of the matrix ATP concentration. Mitochondria were pretreated to alter the matrix adenine nucleotide pool as described under Materials and Methods. The matrix pool was then prelabeled with [$^{14}$C]ADP as usual for the assay of unidirectional efflux in the presence of 1 mM ATP and 2 mM phosphate. Carboxyatractyloside (5 $\mu$M) was present. The matrix [ATP] was calculated from the matrix ATP content and matrix volume was determined separately by the oil separation method after a 1-min incubation as described under Materials and Methods. All measurements were under identical incubation conditions except that external ATP was omitted for determination of matrix ATP content. Matrix ATP was corrected for bound ATP (see text). The solid circles (●) in B are the data in A plotted as $[ATP]_{matrix}$/efflux rate vs $[ATP]_{matrix}$ for determination of $K_m$ and $V_{max}$ in the presence of 1 mM ATP and 2 mM phosphate (see text). B also includes a similar plot for additional experiments in which 2 mM phosphate but no external ATP (○) was included in the efflux assay. Data points are averages of three (○) or four (●) experiments.

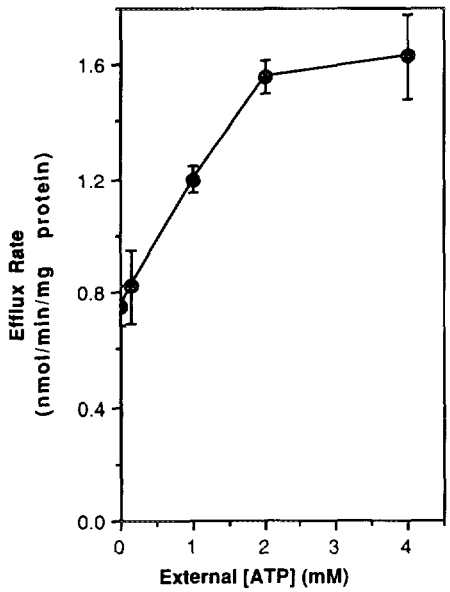


**FIG. 10.** Effect of the external ATP concentration on carboxyatractyloside-insensitive adenine nucleotide efflux. Unidirectional efflux was measured under standard incubation conditions (including 2 mM phosphate and 1 mM ATP) with 5 μM carboxyatractyloside added. The external ATP concentration was varied between 0 and 4 mM. Data are averages of three experiments.

nmol/min/mg with a low ATP/ADP, which suggests that in the presence of $Mg^{2+}$, matrix ATP is preferred over ADP as the substrate for efflux.

In further experiments we determined that $Mg^{2+}$ is required for unidirectional transport. In Fig. 11, influx with 1 mM ATP is plotted as a function of the $Mg^{2+}$ concentration in the medium. The $Mg^{2+}$ requirement appears to be stoichiometric, because the rates of ATP influx follow the theoretical proportion of ATP that is present as ATP–Mg.

In order to confirm the results regarding substrate specificity and $Mg^{2+}$ requirement, we measured the unidirectional efflux of adenine nucleotides that was induced by either 1 mM ATP or 1 mM ADP, in the absence of phosphate. Without phosphate added the mitochondria were unable to convert matrix ADP to ATP and thus the observed rates are due to ADP efflux in exchange for ATP or ADP in the medium. Table III shows that in the presence of $Mg^{2+}$ the ATP-induced rate of efflux is higher than the rate with ADP. In the absence of $Mg^{2+}$, efflux in the presence of ATP is low, which verifies the Mg-dependence for transport that was shown in Fig. 11. However, in the absence of $Mg^{2+}$, the efflux rate induced with 1 mM ADP is almost as high as the rate in the presence of both ATP and $Mg^{2+}$. Hence, ADP seems to be a good substrate for carboxyatractyloside-insensitive transport but only in the absence of $Mg^{2+}$.

When ATP is present in the matrix and in the medium, unidirectional ATP–Mg influx and adenine nucleotide efflux (mainly ATP–Mg) occur at the same time. As presented above, ATP influx and efflux rates depend on the external ATP concentration. Table IV shows that net changes in the adenine nucleotide content of mitochondria measured in the presence of carboxyatractyloside with ATP medium concentrations of 0.15, 1.0, and 2.0 mM are accounted for as the approximate sum of influx and efflux rates measured independently.

### *Characteristics of Carboxyatractyloside-Sensitive Phosphate-Induced Efflux and Comparison with the Carboxyatractyloside-Insensitive Component*

Since we found in the first part of the study that carboxyatractyloside reduces the rates of both net accumulation and net loss of adenine nucleotides (Figs. 4 and 5), we examined an EGTA-insensitive transport component in unidirectional transport experiments in the absence of carboxyatractyloside. Under these circumstances, the activity of the ADP/ATP translocase, which normally mediates rapid one-for-one exchange of adenine nucleotides, obscures a possible carboxyatractyloside-sensitive ATP–$P_i$ exchange. This makes it impossible to have ATP or ADP present in the assay medium. The only way to investigate unidirectional transport in the absence of carboxyatractyloside is by studying phosphate-induced efflux of adenine nucleotides.

In Fig. 12A we titrated phosphate-induced efflux of adenine nucleotides with EGTA in the absence of carboxy-

TABLE II

Adenine Nucleotide Efflux in the Presence and Absence of Oligomycin

| | Efflux (nmol/min/mg protein) | | Matrix amount (nmol/min/mg protein) | |
|---|---|---|---|---|
| | Carboxyatractyloside-insensitive | EGTA-insensitive | ATP | ADP |
| Control | 0.66 ± 0.04 | 0.63 ± 0.06 | 2.93 | 0.43 |
| 12 μg/ml oligomycin | 0.35 ± 0.02 | 0.62 ± 0.14 | 0.97 | 3.17 |

*Note.* Phosphate-induced efflux was measured under standard incubation conditions with 2 mM $P_i$, but no ATP, in the incubation medium in the presence of either 5 μM carboxyatractyloside (carboxyatractyloside-insensitive transport) or 100 μM EGTA (EGTA-insensitive transport). In both cases, efflux was assayed in the presence and absence of oligomycin as indicated. For the assays in which oligomycin was added, the mitochondria were also preincubated with oligomycin at a final concentration of 50 μmg/ml. The ATP and ADP in the matrix under both conditions were determined in separate but identical assays after 1 min incubation. ATP and ADP were assayed in mitochondrial pellets collected by the silicone oil separation method. Bound ATP and ADP (0.50 and 0.54 nmol/mg, respectively, in the control; 0.25 and 0.52 nmol/mg, respectively, in the presence of oligomycin) were subtracted from the measured total amounts to obtain the free ATP and ADP matrix concentrations listed in the table (see text). Values are averages of two experiments.

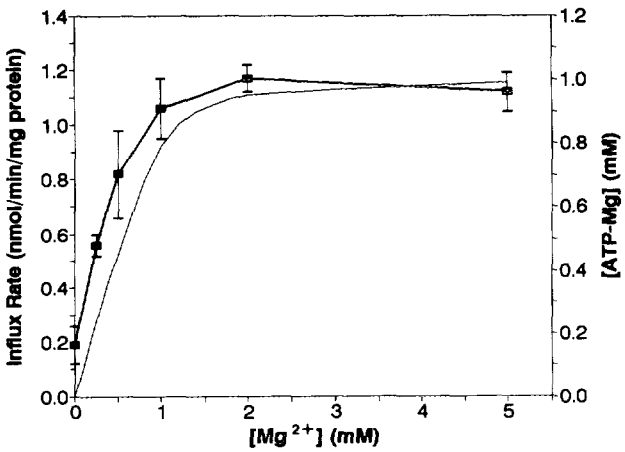


**FIG. 11.** Carboxyatractyloside-insensitive influx as a function of the $Mg^{2+}$ concentration in the medium. Unidirectional influx (in the presence of 5 $\mu$M carboxyatractyloside) was measured with 1 mM ATP and varying $Mg^{2+}$ concentrations from 0 to 5 mM (thick line). Data are plotted as the actual influx rate (solid boxes). All assays included 10 $\mu$M $CaCl_2$ and 1 $\mu$M ruthenium red to maximally activate transport. The thin line shows the fraction of the total ATP that is present as ATP–Mg, calculated from an ATP–Mg dissociation constant of $17.4 \times 10^3$ M$^{-1}$ (9). Data are averages of two experiments.

atractyloside. Maximal inhibition is attained at a concentration of 10 $\mu$M, which should be sufficient to chelate all of the $Ca^{2+}$ in the medium. (Recall from Fig. 7 that micromolar $Ca^{2+}$ is necessary to activate the carboxyatractyloside-insensitive transport of adenine nucleotides). However, EGTA is unable to inhibit phosphate-induced efflux completely. Figure 12B shows that carboxyatractyloside inhibits the rate of efflux only partly as well. Inhibition of phosphate-induced efflux is a function of the carboxyatractyloside concentration; maximal inhibition (about 50%) occurs at a concentration of 800 pmol/mg protein, implying that carboxyatractyloside inhibits at a specific binding site. The data for kidney mitochondria in Table V show that the control rate of efflux (without carboxyatractyloside or EGTA present) is roughly the sum of the separately determined carboxyatractyloside-insensitive and EGTA-insensitive efflux rates. When both components are inhibited by adding both EGTA and carboxyatractyloside, almost no phosphate-induced efflux is observed.

We repeated the experiment in Table V in liver mitochondria, since it has been shown that net transport of adenine nucleotides in liver mitochondria is sensitive to EGTA and insensitive to carboxyatractyloside (6, 8). Table V confirms that there is no EGTA-insensitive rate in liver mitochondria. Phosphate-induced efflux is instead due to carboxyatractyloside-insensitive unidirectional transport. Comparison of carboxyatractyloside-insensitive rates in kidney and liver mitochondria also shows that this EGTA-sensitive transport component is faster in liver than in kidney mitochondria (Table V).

We next studied 200 $\mu$M mersalyl and the absence of $Mg^{2+}$ in the medium for an effect on both components of phosphate-induced efflux. It already has been shown in Table I that carboxyatractyloside-insensitive efflux is completely inhibited by 200 $\mu$M mersalyl. Moreover, in the presence of carboxyatractyloside the same rate of phosphate-induced efflux was observed with and without $Mg^{2+}$ in the medium (Table VI). $Mg^{2+}$ is required for carboxyatractyloside-insensitive ATP transport (as shown above in Table III and Fig. 11) and, under conditions in Table VI, matrix ATP–Mg can be exchanged only for medium phosphate. The data suggest that there is enough $Mg^{2+}$ within the mitochondria to support ATP–Mg efflux because the carboxyatractyloside-insensitive rate was unaffected by the presence or absence of $Mg^{2+}$ in the medium over the 2-min time course. The EGTA-insensitive efflux, however, shows different characteristics. It was only partially inhibited by 200 $\mu$M mersalyl (Table VI); the rate in the absence of $Mg^{2+}$ was inhibited by 30 to 50% in each of three experiments but this inhibition did not reach statistical significance.

With EGTA present, the absence or presence of oligomycin does not affect the rate of phosphate-induced efflux, indicating that the matrix ATP/ADP ratio does not affect rates of EGTA-insensitive adenine nucleotide efflux (Table II).

Figure 13 plots the rate of phosphate-induced efflux for both transport components as a function of the total adenine nucleotide content of the mitochondria. For both kinds of transport, the rate of efflux appears to become saturated at high matrix adenine nucleotide contents. Figure 13 indicates also that $V_{max}$ for EGTA-insensitive transport is lower than for carboxyatractyloside-insensitive transport. We were not able to determine directly

TABLE III

Substrate Specificity and $Mg^{2+}$-Requirement of Carboxyatractyloside-Insensitive Unidirectional Transport

| | Adenine nucleotide efflux (nmol/min/mg protein) |
|---|---|
| 1.0 mM ATP, 5 mM $Mg^{2+}$ | 1.10 ± 0.07 |
| 1.0 mM ATP, no $Mg^{2+}$ | 0.33 ± 0.03 |
| 1.0 mM ADP, 5 mM $Mg^{2+}$ | 0.39 ± 0.01 |
| 1.0 mM ADP, no $Mg^{2+}$ | 0.96 ± 0.05 |

*Note.* Unidirectional efflux was measured in the presence of 5 $\mu$M carboxyatractyloside without phosphate in the incubation medium. Under these conditions, the matrix adenine nucleotides exist predominantly as ADP which could be exchanged for either the ATP or the ADP that was added to the medium. The presence of ATP, ADP, and $Mg^{2+}$ in the assay was varied as indicated. In all assays, 10 $\mu$M $CaCl_2$ and 1 $\mu$M ruthenium red were included to maximally activate transport. When 1 mM ADP was present, 3 mM Ap5A was included to inhibit adenylate kinase. In the absence of $Mg^{2+}$, addition of Ap5A was not necessary, because adenylate kinase requires $Mg^{2+}$; in this case the medium ADP concentration decreased by only 21% after a 2-min incubation (not shown). Values are averages of two experiments.

TABLE IV

Comparison of Carboxyatractyloside-Insensitive Influx, Efflux, and Net Transport under Similar Conditions

| External ATP (mM) | Adenine nucleotide transport rate (nmol/min/mg protein) | | | |
|---|---|---|---|---|
| | Influx | Efflux | Influx + efflux | Measured net transport |
| 0.15 | 0.20 ± 0.01 | −0.82 ± 0.13 | −0.62 | −0.61 ± 0.29 |
| 1.00 | 1.16 ± 0.14 | −1.21 ± 0.23 | −0.05 | −0.07 ± 0.01 |
| 2.00 | 1.84 ± 0.21 | −1.56 ± 0.06 | 0.28 | 0.20 ± 0.10 |

*Note.* Rates for unidirectional influx, efflux, and net transport were measured in the presence of 5 μM carboxyatractyloside under identical incubation conditions. Positive numbers are used to indicate influx; negative numbers indicate efflux. Influx and efflux were determined as initial rates between 15 and 75 s as usual. Measured net transport rates were calculated for initial intervals during which the rates were linear (45 s–4 min for $[ATP]_{ext}$ = 0.15 mM; 45 s–15 min for $[ATP]_{ext}$ = 1 mM and for $[ATP]_{ext}$ = 2 mM). For each ATP concentration the numerical sum of the measured influx and efflux rates (influx + efflux) was close to the actual measured rate of net transport (last column). Values are averages of two experiments.

the preferred substrate for EGTA-insensitive net adenine nucleotide transport.

Figure 14 shows that phosphate-induced efflux for both transport components varies with external phosphate concentrations between 0.5 and 10 mM. At every concentration of phosphate tested, efflux was completely inhibited by the addition of both carboxyatractyloside and EGTA (not shown).

Table VII compares the rate of efflux in the presence of 2 mM phosphate to that with no phosphate in the medium. Since the matrix ATP/ADP is low in the absence of phosphate we inhibited ATP synthetase with oligomycin in all assays. This necessary deenergization is probably the reason for relatively high background rates (i.e., efflux in the presence of both EGTA and carboxyatractyloside) seen in these experiments. In the presence of 100 μM EGTA the efflux rate with 2 mM phosphate is higher than without phosphate (Table VII) which is evidence that phosphate is required for EGTA-insensitive efflux. With 5 μM carboxyatractyloside present, the rate with 2 mM phosphate was 0.39 nmol/min/mg, lower than usual because the mitochondria are deenergized, but the rate without phosphate was lower still (0.23 nmol/min/mg). When taking the background rates (both EGTA and carboxyatractyloside present) into account, these results suggest that the carboxyatractyloside-insensitive transport component is also phosphate dependent.

To gain more evidence that the observed efflux of adenine nucleotides is not due to mitochondrial swelling or nonspecific changes in inner membrane permeability we measured phosphate-induced efflux in the presence of cyclosporin A. Cyclosporin A is known to inhibit mitochondrial swelling and $Ca^{2+}$-induced membrane permeability changes, with a maximal effect at about 150–400 pmol/mg protein in liver (17), heart (18), and kidney mitochondria (19). At a concentration of about 1 mg mito-

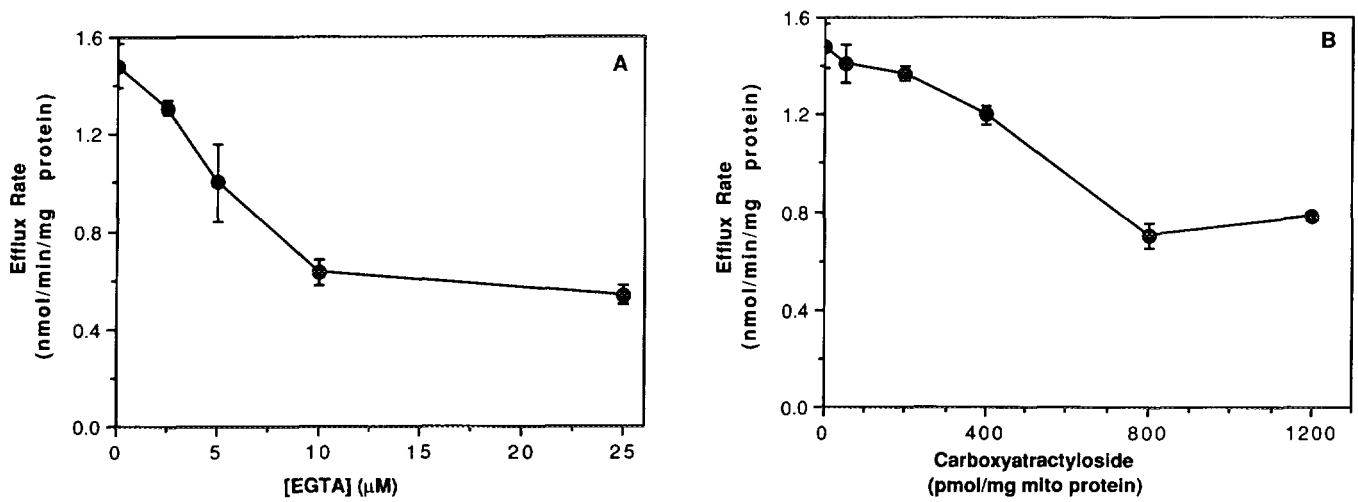


**FIG. 12.** Inhibitor titration of phosphate-induced adenine nucleotide efflux with EGTA and carboxyatractyloside. Efflux was assayed as usual in the presence of 2 mM phosphate but with ATP omitted. Phosphate-induced efflux was titrated with EGTA between 0 and 25 μM (A) and with carboxyatractyloside between 0 and 1200 pmol/mg mitochondrial protein (B). Data are averages of two experiments.

TABLE V

Effect of Carboxyatractyloside and EGTA on Phosphate-induced Adenine Nucleotide Efflux in Kidney and Liver Mitochondria

| | Adenine nucleotide efflux (nmol/min/mg protein) | |
|---|---|---|
| | Kidney | Liver |
| Control | 1.42 ± 0.01 | 2.09 ± 0.03 |
| +5 μM carboxyatractyloside | 0.67 ± 0.06 | 1.92 ± 0.01 |
| +100 μM EGTA | 0.57 ± 0.04 | 0.47 ± 0.04 |
| +5 μM carboxyatractyloside, +100 μM EGTA | 0.07 ± 0.01 | 0.30 ± 0.03 |

*Note.* Phosphate-induced efflux was measured with 2 mM $P_i$ in the absence of external ATP under identical conditions for kidney and liver mitochondria. Values are the average of two experiments.

chondrial protein/ml assay, 1 μM cyclosporin A (1000 nmol/mg protein) had no significant effect on phosphate-induced efflux when neither carboxyatractyloside nor EGTA was present (Table VIII). At extremely high concentrations (10 μM) cyclosporin A inhibited the rate, but only by 25%; this result is in agreement with a recent report showing that micromolar cyclosporin inhibits net adenine nucleotide uptake (20). We also measured net adenine nucleotide loss with 0.15 mM ATP in the incubation medium in the presence of 1 and 10 μM cyclosporin A and net uptake with 2 mM ATP in the presence of 1 μM cyclosporin A. In none of these cases did cyclosporin A have any effect on rates of net transport (data not shown).

## DISCUSSION

This study investigated the mechanism by which the matrix adenine nucleotide pool size is regulated in isolated kidney mitochondria. The pool size decreases *in vivo* during ischemia and then subsequently recovers during reperfusion (1, 2), and we hypothesized that a mitochondrial adenine nucleotide transport process must be responsible. Prior to this work, the only well-characterized adenine

TABLE VI

Effects of $Mg^{2+}$ and Mersalyl on Phosphate-Induced Adenine Nucleotide Efflux in the Presence of Carboxyatractyloside or EGTA

| | Efflux (nmol/min/mg protein) | |
|---|---|---|
| | Carboxyatractyloside-insensitive | EGTA-insensitive |
| $+Mg^{2+}$ | 0.59 ± 0.09 | 0.64 ± 0.15 |
| $-Mg^{2+}$ | 0.68 ± 0.07 | 0.40 ± 0.12 |
| $+Mg^{2+}$, +200 μM mersalyl | 0.03 ± 0.02 | 0.30 ± 0.09 |

*Note.* Efflux was measured with 2 mM $P_i$ and no ATP in the incubation medium. When examining carboxyatractyloside-insensitive transport, 5 μM carboxyatractyloside was included ($n$ = 2). When examining EGTA-insensitive transport, 100 μM EGTA was present ($n$ = 3). In assays with 5 μM carboxyatractyloside, a Ca-EGTA buffer system was included to set $[Ca^{2+}]_{free}$ at 4 μM because $Mg^{2+}$ was a variable (see text).

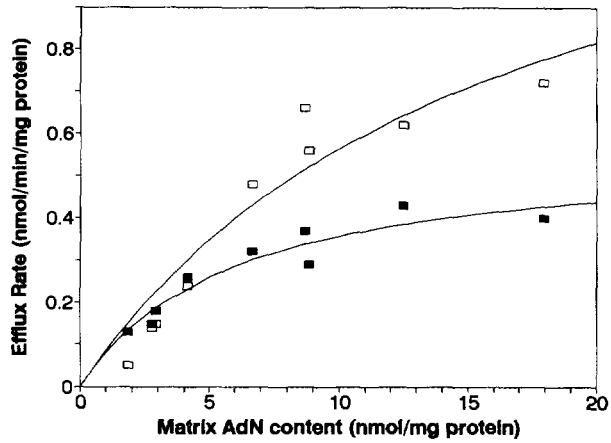


**FIG. 13.** Carboxyatractyloside-insensitive and EGTA-insensitive phosphate-induced efflux of adenine nucleotides as a function of the matrix adenine nucleotide content. The matrix adenine nucleotide (AdN) pool size was altered before efflux rates were measured, as described in the legend to Fig. 9. Efflux rates were determined in the presence of 2 mM phosphate with no external ATP and in the presence of either 5 μM carboxyatractyloside (□) or 100 μM EGTA (■). Data are averages of three experiments. The fitted curves were determined by nonlinear regression (21).

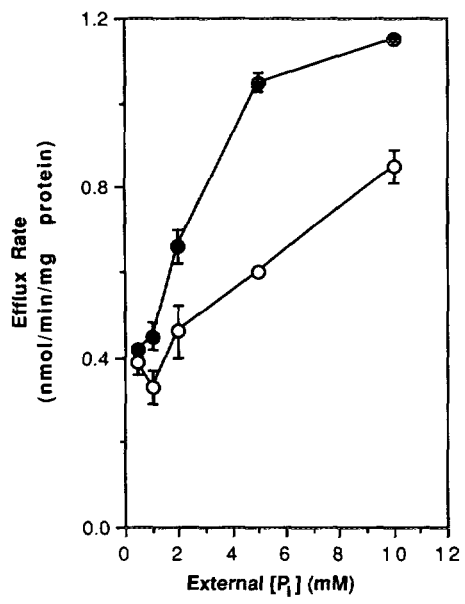


**FIG. 14.** Effect of external phosphate concentration on efflux of adenine nucleotides. Unidirectional efflux was assayed as usual except that external ATP was omitted and the phosphate concentration in the medium was varied between 0.5 and 10 mM. Rates were determined in the presence of 5 μM carboxyatractyloside (●) or 100 μM EGTA (○). Data are averages of two experiments.

TABLE VII

Adenine Nucleotide Efflux with No External ATP in the Presence and Absence of Phosphate

| | Efflux (nmol/min/mg protein) |
|---|---|
| 2 mM $P_i$, 100 μM EGTA | 0.58 ± 0.03 |
| No $P_i$, 100 μM EGTA | 0.21 ± 0.07 |
| 2 mM $P_i$, 5 μM carboxyatractyloside | 0.39 ± 0.04 |
| No $P_i$, 5 μM carboxyatractyloside | 0.23 ± 0.08 |
| 2 mM $P_i$, 100 μM EGTA, 5 μM carboxyatractyloside | 0.18 ± 0.08 |
| No $P_i$, 100 μM EGTA, 5 μM carboxyatractyloside | 0.27 ± 0.03 |

*Note.* Adenine nucleotide efflux was measured under the various conditions indicated in the table. Mitochondria were preincubated with 50 μg/ml oligomycin and oligomycin (12 μg/ml) was present in all assays to prevent variations in the matrix ATP/ADP ratio that might have occurred among the different assay conditions. Values are the average of two experiments.

nucleotide transport function in kidney mitochondria was the one-for-one exchange of ADP and ATP that occurs over the ADP/ATP translocase. Obviously, one-for-one exchange cannot bring about net increases or decreases in the matrix adenine nucleotide content. There was some evidence that isolated kidney mitochondria could take up adenine nucleotides by an alternate pathway (2, 20), and the primary objective of this research was to characterize net transport in detail. We reasoned that the mechanism of net adenine nucleotide transport in kidney mitochondria might be similar to net transport in liver mitochondria which occurs over the well-characterized ATP–Mg/$P_i$ carrier (3). It was therefore somewhat surprising to find two independent mechanisms for net adenine nucleotide transport in kidney mitochondria. One of the two transport mechanisms is analogous to that of the ATP–Mg/$P_i$ carrier in liver mitochondria; it is regulated by micromolar $Ca^{2+}$ and is not sensitive to carboxyatractyloside. The other mechanism is not regulated by $Ca^{2+}$ and is inhibited by carboxyatractyloside. Both transport mechanisms allow changes in the matrix adenine nucleotide content by transport in exchange with phosphate, but their kinetic properties and regulation are quite different, as the following discussion shows.

Net transport was characterized first in the absence of any inhibitors. Net uptake was observed when the external ATP concentration was 1 or 2 mM. Assuming that matrix ATP (2.93 nmol/mg protein) was distributed into the measured matrix volume, the matrix ATP concentration was initially about 3.0 mM and so net uptake of ATP occurred against a concentration gradient. Net loss of matrix adenine nucleotides occurred when the external concentration of ATP was lower than 1 mM. With 0.15 mM ATP, a steady state was attained when the calculated matrix ATP concentration was 0.72 mM, suggesting that transport in one direction was equal to that in the other when the concentration gradient was about 4.8 in/out. This is similar to the steady state distribution of adenine nucleotides in liver mitochondria, which is established in proportion to the phosphate concentration gradient by exchange of ATP–Mg for phosphate (3).

Both net uptake and net loss of adenine nucleotides in kidney mitochondria were inhibited by mersalyl, as is also true for net transport in liver mitochondria (6). However, in kidney mitochondria, net transport was partially inhibited by carboxyatractyloside, whereas in liver mitochondria carboxyatractyloside has no direct effect on transport. The sensitivity to EGTA was also different between liver and kidney mitochondria. For liver, EGTA inhibits net uptake and net loss completely because the ATP–Mg/$P_i$ carrier requires micromolar $Ca^{2+}$ for activation (8). For kidney, net loss was only partly inhibited by EGTA; the EGTA-sensitive and carboxyatractyloside-sensitive rates of net loss were additive and the addition of both inhibitors at the same time completely blocked net loss. However, EGTA alone completely inhibited net uptake with 2 mM ATP even when oligomycin was included to diminish the transmembrane [ATP–Mg] gradient. This result was complicated since (as noted above) carboxyatractyloside alone partially inhibited net uptake and the effects of the two inhibitors on net uptake therefore were not additive. Nevertheless, the sensitivity of net loss and net uptake to both EGTA and carboxyatractyloside suggested two distinct mechanisms for adenine nucleotide transport in kidney mitochondria. These mechanisms were studied further and separately by measuring the unidirectional rates of transport in the presence of one inhibitor or the other.

Unidirectional adenine nucleotide influx and efflux were studied first in the presence of carboxyatractyloside. The isolated carboxyatractyloside-insensitive influx and efflux rates were inhibited completely by EGTA or mersalyl. Sensitivity to EGTA suggested regulation by calcium, which was investigated in detail. Influx and efflux varied as a hyperbolic function of external $[Ca^{2+}]_{free}$ between 0.3 and 2 μM. The apparent $K_d$ and $V_{max}$ were estimated by nonlinear regression (21) as 2.1 μM and 1.74 nmol/min/mg protein for efflux, and 1.5 μM and 1.6 nmol/min/mg

TABLE VIII

Effect of Cyclosporin A on Phosphate-Induced Adenine Nucleotide Efflux

| | Efflux (nmol/min/mg protein) |
|---|---|
| Control | 1.34 ± 0.06 |
| +1 μM cyclosporin A | 1.30 ± 0.06 |
| +10 μM cyclosporin A | 1.00 ± 0.09 |

*Note.* Phosphate-induced adenine nucleotide efflux was measured under standard incubation conditions with 2 mM $P_i$ and no external ATP. Neither carboxyatractyloside nor EGTA was present in the assays. Values are the average of two experiments.

protein for influx. The similar calcium dependence for influx and efflux suggests that the unidirectional transport components are tightly coupled, probably as an exchange mechanism. The calcium dependence also suggests that the carboxyatractyloside-insensitive adenine nucleotide transport pathway might be subject to hormonal control.

The carboxyatractyloside-insensitive influx rate varied as a function of the external ATP concentration, and the efflux rate varied as a function of the matrix ATP concentration. The efflux rate also varied as a function of the external ATP concentration, supporting the idea that influx and efflux are coupled processes. It was interesting to note that when external ATP and phosphate were both 2 mM, efflux was maximal, suggesting that the matrix adenine nucleotide concentration rather than external counterions was rate-limiting for efflux under these conditions. Under standard conditions the concentration of external ATP required for half-maximal influx was 1.7 mM. The matrix ATP concentration required for half-maximal efflux was much higher, 8.6 mM. The apparent $K_m$ values were different on each side of the membrane, probably due to the difference in phosphate concentration. Phosphate is a competitive substrate for transport. The external phosphate concentration is 2 mM under standard conditions while the matrix phosphate concentration is severalfold higher because a concentration gradient is maintained by the $P_i$/OH carrier. $Mg^{2+}$ was required in stoichiometric amounts for ATP influx and ATP-induced adenine nucleotide efflux. Therefore carboxyatractyloside-insensitive ATP transport on this carrier probably occurs as the ATP–Mg chelate. Adenine nucleotide efflux occurred at a much slower rate when the matrix ATP/ADP was lowered with oligomycin, suggesting that ATP–Mg is a much better substrate than ADP. Moreover, in the presence of oligomycin, phosphate-induced efflux (presumably ADP efflux) was insensitive to added $Mg^{2+}$. $Mg^{2+}$ was required for ATP-induced efflux, but not for ADP-induced efflux, and, in fact, ADP-induced efflux was slower in the presence vs absence of $Mg^{2+}$. These data establish that $Mg^{2+}$ is required for ATP movements but not for ADP movements on the carrier.

All of the aforementioned characteristics for carboxyatractyloside-insensitive transport in kidney mitochondria are very similar to those of the transport mechanism described previously for the ATP–Mg/$P_i$ carrier in liver mitochondria, and we conclude that the same transport process is responsible for $Ca^{2+}$-regulated adenine nucleotide movements in both tissues. Under standard conditions, both the $K_m$ and the $V_{max}$ for ATP–Mg influx are lower by about half in kidney compared to liver, and the matrix adenine nucleotide content is also lower by half. Values are: for kidney, $K_m$ is 1.7 mM, $V_{max}$ is 3.5 nmol/min/mg protein, adenine content is 7–8 nmol/mg protein; for liver (6), $K_m$ is 2.7 mM, $V_{max}$ is 8.3 nmol/min/mg protein, adenine content is 12–14 nmol/mg protein. In liver and most likely in kidney as well, transport probably occurs as an electroneutral exchange of divalent anion substrates; physiologically, ATP–$Mg^{2-}$ and $HPO_4^{2-}$ are the most important substrates, although $HADP^{2-}$ may be transported under extreme circumstances when the $[HADP^{2-}]_{free}$ is high enough (22, 23). Net adenine nucleotide transport occurs when the rate of ATP–$Mg^{2-}$ or $HADP^{2-}$ exchange for $HPO_4^{2-}$ in one direction exceeds exchange in the opposite direction, and steady state will be attained when the opposing exchange rates are equal.

As explained under Results, the carboxyatractyloside-sensitive pathway for net adenine nucleotide movements was studied in the presence of EGTA to distinguish it from the ATP–Mg/$P_i$ carrier activity. Because carboxyatractyloside was of course omitted, adenine nucleotides could not be included in the external medium and the unidirectional kinetics of net transport could be studied only as a phosphate-induced [$^{14}$C]adenine nucleotide efflux. Using this specific assay, titration with EGTA rigorously confirmed the existence of a residual EGTA-insensitive rate of efflux that was inhibited by carboxyatractyloside. Conversely, titration with carboxyatractyloside distinguished a residual carboxyatractyloside-insensitive rate that was inhibited by EGTA. Both types of phosphate-induced adenine nucleotide efflux varied as a function of the external phosphate concentration and as a function of the matrix adenine nucleotide concentration. Efflux was not attributable to nonspecific leakage and it was not affected by cyclosporin A. All the evidence presented is consistent with a specific exchange of external phosphate with matrix adenine nucleotide that occurs additively via two pathways; one most certainly is the ATP–Mg/$P_i$ carrier as discussed above, the other is a novel carboxyatractyloside-sensitive pathway.

In addition to their opposite sensitivities to EGTA and carboxyatractyloside, other differences between the two types of phosphate-induced efflux were observed. In contrast to EGTA-sensitive efflux, EGTA-insensitive efflux was only partially (versus completely) inhibited by mersalyl, was partially inhibited by omitting $Mg^{2+}$ (versus unaffected), and was unaffected (versus inhibited) by a decrease in the matrix ATP/ADP ratio.

Some of the data support the idea that EGTA-insensitive adenine nucleotide transport represents a novel function of the ADP/ATP translocase. For example, the amount of carboxyatractyloside required for maximal inhibition is 800 pmol/mg mitochondrial protein, in the range of values (e.g., 375 pmol/mg mitochondrial protein) determined by inhibitor titration of kidney ADP/ATP translocase (1). Carboxyatractyloside is thought to be a very specific inhibitor of the ADP/ATP translocase, but the idea that it might inhibit another adenine nucleotide transport protein remains a formal possibility. Perhaps the most striking argument against a novel function of the extant ADP/ATP translocase is the fact that carboxyatractyloside-sensitive phosphate-induced adenine nucleotide efflux could not be demonstrated in liver mitochondria. An interesting possibility is that the car-

boxyatractyloside-sensitive component of ATP–Mg/$P_i$ exchange might be an alternate function of an isoform of ADP/ADP translocase that is expressed in kidney (and heart) but not in liver. The translocases in heart and liver are antigenically distinct (24). There are three genes for translocase that are developmentally and differentially expressed (25, 26). In mouse, transcripts for two of the genes (*ANT1* and *ANT3*) are much more abundant in kidney and heart compared to liver (26). In isolated heart mitochondria, net efflux of adenine nucleotides is observed in medium containing either pyrophosphate or phosphate. Transport is inhibited by carboxyatractyloside, and the mechanism is not well understood. It has some characteristics in common with the carboxyatractyloside-sensitive component of net transport described here for kidney mitochondria and is clearly different than transport on the ATP–Mg/$P_i$ carrier (4, 5, 27, 28).

In summary, this study demonstrates that net adenine nucleotide transport in kidney mitochondria occurs by two distinct mechanisms. Both involve exchange for phosphate; one mechanism is identical to that of the ATP–Mg/$P_i$ carrier described for liver, the other either represents a novel function of the ADP/ATP translocase or another carboxyatractyloside-sensitive carrier. Both mechanisms contribute to the regulation of the matrix adenine nucleotide pool size, although differences in $V_{max}$ suggest that the ATP–Mg/$P_i$ carrier is the more important of the two. Also, it appears that carboxyatractyloside-sensitive net transport is only important for net adenine nucleotide loss, since, in the absence of inhibitors, all net uptake is accounted for as EGTA-sensitive transport.

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## REFERENCES

1. Henke, W., and Jung, K. (1991) *Biochim. Biophys. Acta* **1056,** 71–75.
2. Henke, W., and Nickel, E. (1992) *Biochim. Biophys. Acta* **1098,** 233–239.
3. Aprille, J. R. (1988) *FASEB J.* **2,** 2547–2556.
4. Asimakis, G. K., and Conti, V. R. (1985) *Am. J. Physiol.* **249,** H1009–H1016.
5. Henke, W., Ziegler, M., Dubiel, W., and Jung, K. (1991) *Adv. Exp. Med. Biol.* **309,** 309–312.
6. Austin, J., and Aprille, J. R. (1984) *J. Biol. Chem.* **259,** 154–160.
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
8. Nosek, M. T., Dransfield, D. T., and Aprille, J. R. (1990) *J. Biol. Chem.* **265,** 8444–8450.
9. Bartfai, T. (1979) *Adv. Cyclic Nucleotide Res.* **10,** 219–242.
10. Lukacs, G. L., and Kapus, A. (1987) *Biochem. J.* **248,** 609–613.
11. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260,** 3440–3450.
12. Austin, J., and Aprille, J. R. (1983) *Arch. Biochem. Biophys.* **222,** 321–325.
13. Lamprecht, W., and Trautschold, I. (1974) *in* Methods of Enzymatic Analysis (Bergmeyer, H. U., Ed.), Vol 4, pp. 2101–2110, Verlag-Chemie, Weinheim/Academic Press, New York.
14. Jaworek, D., Gruber, W., and Bergmeyer, H. U. (1974) *in* Methods of Enzymatic Analysis (Bergmeyer, H. U., Ed.), Vol. 4, pp. 2127–2131, Verlag-Chemie, Winheim/Academic Press, New York.
15. Aprille, J. R., and Austin, J. (1981) *Arch. Biochem. Biophys.* **212,** 689–699.
16. Austin, J., and Aprille, J. R. (1983) *Arch. Biochem. Biophys.* **222,** 321–325.
17. Broekemeier, K. M., Dempsey, M. E., and Pfeiffer, D. R. (1989) *J. Biol. Chem.* **264,** 4826–4830.
18. Halestrap, A. P., and Davidson, A. M. (1990) *Biochem. J.* **268,** 153–160.
19. Strzelecki, T., Kumar, S., Khauli, R., and Menon, M. (1988) *Kidney Int.* **34,** 234–240.
20. Henke, W., Nickel, E., and Jung, K. (1992) *Biochem. Pharmacol.* **43,** 1021–1024.
21. Holzhütter, H. G., and Colosimo, A. (1990) *Comput. Appl. Biosci.* **6,** 23–28.
22. Joyal, J. L., and Aprille, J. R. (1992) *J. Biol. Chem.* **267,** 19198–19203.
23. Nosek, M. T., and Aprille, J. R. (1992) *Arch. Biochem. Biophys.* **296,** 691–697.
24. Schultheiss, H.-P., and Klingenberg, M. (1985) *Arch. Biochem. Biophys.* **239,** 273–279.
25. Lunardi, J., Hurko, O., Engel, W. K., and Attardi, G. (1992) *J. Biol. Chem.* **267,** 15267–15270.
26. Stepien, G., Torroni, A., Chung, A. B., Hodge, J. A., and Wallace, D. C. (1992) *J. Biol. Chem.* **267,** 14592–14597.
27. Asimakis, G. K., Sandhu, G. S., Conti, V. R., Sordah, L. A., and Zwischenberger, J. B. (1990) *Circ. Res.* **66,** 302–310.
28. Wilson, D. E., and Asimakis, G. K. (1987) *Biochim. Biophys. Acta* **893,** 470–479.