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ADENINE NUCLEOTIDE TRANSPORT IN SONIC SUBMITOCHONDRIAL PARTICLES

KINETIC PROPERTIES AND BINDING OF SPECIFIC INHIBITORS

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SUMMARY

1. A procedure for preparation of sonic submitochondrial particles competent for adenine nucleotide transport is described. ADP or ATP transport was assayed, in the presence of oligomycin, in a saline medium made of 0.125 M KCl, 1 mM EDTA, 10 mM 4-morpholinopropane sulfonic acid buffer, pH 6.5.

2. Sonic particles transport ADP and ATP by an exchange diffusion process. Externally added ADP (or ATP) is exchanged with internal ADP and ATP with a stoichiometry of one to one. The V value for ADP transport at 5 °C was between 2 and 3 nmol/min per mg protein.

3. The transport system in sonic particles is specific for ADP and ATP. It is strongly dependent on temperature. The activation energy between 0 and 9 °C is approx. 35 kcal/mol. The optimum pH is 6.5.

4. Like in intact mitochondria, externally added ADP is transported into sonic particles faster at a given concentration than externally added ATP. The V value for ADP transport is 1.5–2 times higher than the V value for ATP transport.

5. The transition from the energized to the deenergized state in sonic particles results in a decrease of the pH gradient across the membrane (internal pH < external pH) and in a 2–4-fold increase in the K_m value for ATP. This latter effect is opposite to that found for transport of added ATP in intact mitochondria (Souverijn, J. H. M., Huisman, L. A., Rosing, J. and Kemp, Jr., A. (1973) Biochim. Biophys. Acta 305, 185–198). Energization has no effect on the V value of ATP transport in sonic particles.

6. In contrast to intact mitochondria, inhibition of ADP transport in sonic particles by bongkrekic acid does not have any lag-time and does not depend on pH. The inhibition caused by bongkrekic acid is a mixed type inhibition with a K_i value of 1.2 μM. Atractyloside and carboxyatractyloside do not inhibit ADP transport in sonic particles, unless the particles have been preloaded with these inhibitors during the sonication.

Abbreviations: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; MOPS, 4-morpholinopropane sulfonic acid; EDTA, ethylenediamine tetraacetic acid.

7. Palmityl-CoA added to sonic particles inhibits efficiently ADP transport. The mixed type inhibition found with palmityl-CoA has a K_i value of 1.6 μM.

8. [^{3}H]Bongkrekic acid binds to sonic particles readily and with high affinity. Bongkrekic acid binding to sonic particles does not depend on pH and it has a saturation plateau, corresponding approximately to 1.3 mol of site per mol of cytochrome *a*. The number of [^{3}H]atractyloside binding sites is much lower (one-fifth of the bongkrekic acid). External carboxyatractyloside does not compete with [^{3}H]bongkrekic acid for binding to sonic particles. However, when carboxyatractyloside is present inside the particles, it inhibits the binding of [^{3}H]bongkrekic acid.

INTRODUCTION

Kinetic and topological properties of the adenine nucleotide carrier in mitochondria have been studied in detail in the last ten years (for review cf. ref. 1). A remarkable property of this carrier is its asymmetric behaviour in intact mitochondria, concerning both (1) the transport itself (preferential ADP_{ex}/ATP_{in} exchange leading to a higher ATP/ADP ratio outside than inside mitochondria [2–4] and (2) the binding of specific inhibitors (atractyloside on the outside, bongkrekic acid on the inside [5, 6]). Because submitochondrial particles obtained by sonication of heart mitochondria (sonic particles have their membrane reversed with respect to that of intact mitochondria [7]), they may be used (1) to verify whether there is a binding asymmetry of the ADP carrier for specific inhibitors, like atractyloside and bongkrekic acid; (2) to determine to what extent the preferential exchange of ADP_{ex} for ATP_{in} is governed by a membrane potential and/or a pH gradient.

In this paper, we describe a procedure for the preparation of sonic particles which are competent for adenine nucleotide transport. We also report on the kinetic properties of adenine nucleotide transport in sonic particles and about its specificity for ADP and ATP. Finally the inhibitory effect of atractyloside, carboxyatractyloside and bongkrekic acid on ADP transport has been analyzed in detail and related to the binding properties of the corresponding radio-labeled inhibitors. It was found that sonic particles and intact mitochondria have similar kinetic and specificity properties. However, they differ by the effect of specific inhibitors and of uncouplers on the transport of ADP and ATP and by the binding properties of labeled atractyloside and bongkrekic acid. Some of the findings described here have been presented in a preliminary communication [8].

METHODS

Beef heart mitochondria were prepared according to Smith [9] and frozen overnight at −20 °C. After thawing, they were submitted to sonication according to the procedure described by Beyer [10]. As reported by Beyer [10] the overnight storage of mitochondria at −20 °C increased the yield in sonic particles. Standard submitochondrial particles competent for ADP or ATP transport were prepared by sonication of the thawed mitochondria, after dilution to a concentration of 10–15 mg protein/ml in 0.25 M sucrose, 3 mM Tris buffer, 15 mM $MgCl_2$, 5 mM ADP and 5 μg oligomycin/mg protein at pH 7.4 (standard sonication medium). The mitochondrial suspension was exposed to successive sonic oscillations in a Branson sonifier at

90–100 W for six periods of 30 s each, separated by 1-min intervals. The temperature of the suspension was maintained at 2–5 °C. Unbroken mitochondria were removed by centrifugation at 15 000 rev./min (Spinco rotor 30) for 15 min. The sonic particles were recovered from the supernatant by centrifugation at 30 000 rev./min (Spinco rotor 30) for 60 min. After washing in 0.25 M sucrose plus 3 mM Tris buffer, pH 7.4, the final pellet was resuspended in the same medium. As shown by electron microscopy of negatively stained particles, at least 90 % of the particles had an inverted inner membrane in which the stalked particles corresponding to the F_1-ATPase were facing the medium [11]. In some specific cases, to eliminate externally bound nucleotides, the vesicles were passed through Dowex 1-X2, an anion-exchange resin [12].

Adenine nucleotide transport was routinely assayed by the direct exchange procedure at 5 °C [13]. Sonic particles (about 1 mg protein) were dispensed into 10 ml round-bottom centrifuge tubes with 0.125 M KCl, 1 mM EDTA, 10 mM MOPS buffer, pH 6.5, and 5 μg oligomycin, final volume 1 ml (standard incubation medium). The transport reaction was initiated by addition of [^{14}C]ADP or [^{14}C]-ATP. Unless otherwise stated the final concentration of [^{14}C]ADP or [^{14}C]ATP was 100 μM and the incubation period 15 s. The transport reaction was stopped by addition of 10 μM bongkrekic acid, followed immediately by centrifugation in a Sorvall SSl centrifuge at 25 000 $\times g$ for about 10 min at 0 °C. The saline medium permitted a more rapid sedimentation of the particles than a sucrose medium. The pellets were digested in 1 ml of formamide at 180 °C and their radioactivity was determined by liquid scintillation. In order to take account of the non-specifically bound radioactivity and to make appropriate corrections, a "zero" incubation was carried out with 10 μM bongkrekic acid added to the sonic particles before addition of [^{14}C] ADP or [^{14}C]ATP. The rate of transport was calculated as previously described [13]. When indicated, ADP transport was studied by the reverse or back exchange technique [13] with particles preloaded with labeled adenine nucleotides. In specific cases, incubation was terminated by rapid filtration through a 0.45 μm Millipore filter inserted in a filter holder fitted to a syringe and the radioactivity of the filtrate was assayed. Nearly all of the particles were retained by the filter.

Adenine nucleotides were assayed enzymatically according to Adam [14, 15] from neutralized perchloric extracts.

The pH gradient across the membrane of sonic particles was determined with [^{14}C]methylamine [16] added to the particles incubated in the standard incubation medium (see above). Accordingly, additions were made to energize (succinate) or to deenergize (FCCP) the particles. In a few assays pH was adjusted to 7.5.

[^{3}H]Atractyloside and [^{3}H]bongkrekic acid were prepared according to procedures described in refs. 17 and 6. Binding assays are described in the legends of Figs. 9 and 10.

The internal space of the sonic particles was measured as sucrose impermeable space with [^{14}C]sucrose and $^{3}H_2O$. The extra-particle space was calculated from the ^{14}C radioactivity of the pellet after centrifugation and the total space from the ^{3}H radioactivity. The cytochrome *a* content of the particles was determined according to Van Gelder and Muijers [18].

RESULTS

Adenine nucleotide content of sonic particles

Standard sonic particles, loaded with adenine nucleotides by sonication of beef heart mitochondria in the standard sonication medium in the presence of 5 mM ADP (see Methods), contained 2.8–3.0 nmol of ADP plus ATP and 1.5–2.0 nmol of AMP per mg protein (Table I). This corresponded to a concentration of total adenine nucleotides of 5–10 mM, based on an internal space volume of 1–0.5 μl per mg protein. Replacement of ADP by ATP in the sonication medium resulted in a similar nucleotide distribution, but replacement of $MgCl_2$ by EDTA led to a decrease of the total amount of ADP plus ATP in the particles. Increasing the ADP concentration in the standard sonication medium resulted in an increase of the content of the particles in ADP and ATP (Fig. 1). This increased loading capacity was independent of temperature at least between 0 and 12 °C during sonication.

Standard particles were able to retain their adenine nucleotide pool without leakage for at least 24 h at 0 °C and even for longer periods of time (at least 6 months) when frozen in liquid nitrogen. The ADP/ATP distribution in standard particles did not change when the particles were energized by aerobic incubation with 2 mM succinate and 5 μg oligomycin/mg protein, or deenergized by addition of 1 μM FCCP, or when carboxyatractyloside was added to the sonication medium.

Effect of the concentration of internal ADP plus ATP on the rate and extent of [^{14}C]-ADP uptake in sonic particles

[^{14}C]ADP added to sonic standard particles preloaded with adenine nucleotides was readily taken up by the particles. As [^{14}C]ADP uptake is inhibited by bongkrekic acid (see below), addition of bongkrekic acid was used as a means to stop [^{14}C]ADP uptake. The rate of [^{14}C]ADP uptake was proportional to the ADP plus ATP content of the particles (Fig. 2). This holds also for the extent of [^{14}C]ADP

TABLE I

DISTRIBUTION OF ADENINE NUCLEOTIDES IN STANDARD SONIC PARTICLES ($MgCl_2$ PARTICLES) AND IN EDTA PARTICLES. RATE AND EXTENT OF [^{14}C]ADP UPTAKE

The rate of exchange was measured by direct uptake of [^{14}C]ADP at 0 °C (cf. Methods). The extent of [^{14}C]ADP uptake represents the amount of ^{14}C radioactivity incorporated for 40 min at 0 °C. EDTA particles were prepared from beef heart mitochondria in the same medium as that used for the standard $MgCl_2$ particles except that $MgCl_2$ was replaced by 2 mM EDTA.

	Internal adenine nucleotide particles (nmol/mg)			Rate of [^{14}C]-ADP uptake (nmol/mg per min)	Extent of [^{14}C]ADP uptake (nmol/mg)
	ADP	ATP	AMP		
Standard $MgCl_2$ particles	1.6±0.2*(14)**	1.2±0.2 (14)	1.8±0.3 (14)	0.5±0.1 (4)	1.0±0.1 (4)
EDTA particles	0.9, 1.1 (2)**	1.2, 1.3 (2)	1.0, 1.0 (2)	0.1, 0.1 (2)	0.20, 0.16 (2)

* Standard error.

** Number of assays.

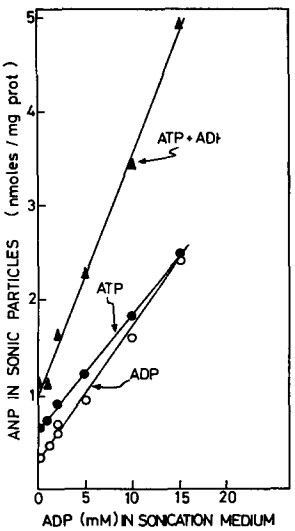

Fig. 1. Effect of the adenine nucleotide concentration of the sonication medium on the adenine nucleotide content of sonic particles. Standard sonic particles were prepared in the presence of increasing concentrations of ADP as described in Methods. ADP and ATP in the particles were assayed enzymatically.

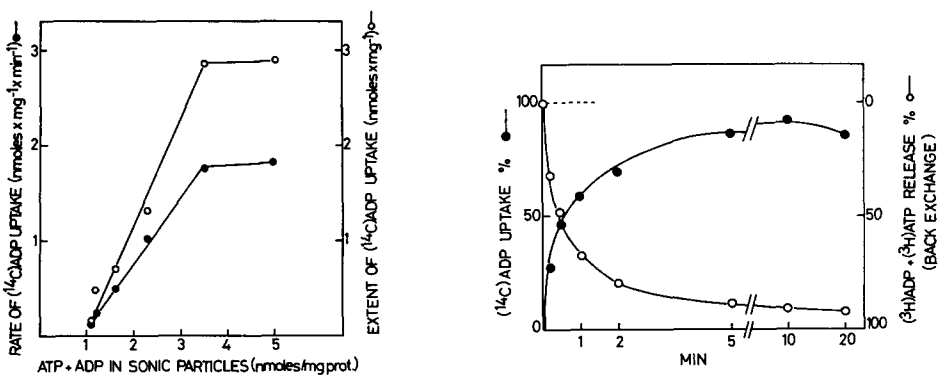

Fig. 2. Rate and extent of [^{14}C]ADP uptake in sonic particles as a function of their adenine nucleotide content. The extent of exchange represents the amount of ^{14}C radioactivity which was found in sonic particles incubated with [^{14}C]ADP for 40 min at 5 °C. The rate of [^{14}C]ADP uptake was assayed as described in Methods. The non-specifically bound radioactivity was corrected by running a "zero" incubation with bongkrekic acid added before [^{14}C]ADP (cf. Methods).

Fig. 3. Stoichiometry of adenine nucleotide exchange in sonic particles. Standard sonic particles (60 mg protein) in 5 ml of 0.25 M sucrose, 1 mM EDTA, 10 mM MOPS, pH 6.5, and 5 μg/ml oligomycin were loaded at 5 °C for 40 min with [^{3}H]ADP (50 μM final concentration). Then the particles were diluted 4-fold with the same medium without [^{3}H]ADP and sedimented by centrifugation for 60 min at 30 000 rev./min (Spinco rotor 30). After washing once with the above medium, the pellet was resuspended to give a final concentration of 10 mg protein/ml. Aliquot fractions of the particle suspension (1 mg) were distributed in centrifuge tubes for transport assay (back exchange procedure, see Methods). The reaction was initiated by addition of [^{14}C]ADP (final concentration 100 μM) and stopped by addition of 10 μM bongkrekic acid, followed by centrifugation.

uptake, which designates the amount of ^{14}C radioactivity incorporated for 40 min (Fig. 2). The ^{14}C radioactivity was roughly distributed as follows: 40–50 % [^{14}C]-AMP, 60–50 % [^{14}C]ADP plus [^{14}C]ATP. Accumulation of [^{14}C]AMP, in spite of the presence of EDTA in the incubation medium, may be due to a residual adenylate kinase activity bound or entrapped in the particles. A significant fraction of [^{14}C]-AMP was probably bound on the outer surface of the particles, since treatment of sonic particles by Dowex 1-X2, an anion-exchange resin, decreased by 3–4 times the content of the particles in [^{14}C]AMP without altering the amount of [^{14}C]ADP and [^{14}C]ATP. Replacement of $MgCl_2$ in the sonication medium by EDTA strikingly decreased both the rate and extent of [^{14}C]ADP uptake (Table I).

The extent of [^{14}C]ADP uptake was limited, reaching a maximum at about 3 nmol per mg protein. It was pertinent to know whether this limited uptake of [^{14}C]ADP was due to binding or transport. Subsequent experiments have demonstrated that [^{14}C]ADP uptake is mainly accounted for by transport.

Kinetics of transport. Specificity and effect of inhibitors

As shown in the double-labeling experiment in Fig. 3 [^{14}C]ADP added to sonic vesicles preloaded with [^{3}H]ADP was exchanged against the internal [^{3}H]-ADP plus [^{3}H]ATP. In this experiment we started from sonic particles which were loaded with [^{3}H]adenine nucleotides during a preincubation period with [^{3}H]ADP. Upon addition of [^{14}C]ADP, the distribution of the [^{3}H]adenine nucleotides released to the medium was 10 % [^{3}H]ATP 72 % [^{3}H]ADP and 18 % [^{3}H]AMP. As AMP is not transported in sonic vesicles (see below), the presence of [^{3}H]AMP in the external medium was not taken into account. The rate at which [^{3}H]ADP and [^{3}H]ATP were released was equal to the rate at which [^{14}C]ADP was taken up. Furthermore, the content of the particles in ADP plus ATP (enzymatically determined) remained constant. These findings taken together indicate that the added [^{14}C]ADP is exchanged against the internal [^{3}H]ADP plus [^{3}H]ATP, and that the stoichiometry of the exchange is one.

The rate of exchange depended on pH and temperature. It was twice faster at pH 7.5 than at pH 6.5. The activation energy, calculated from an Arrhenius plot was close to 35 kcal/mol between 0 and 9 °C, a value similar to that found for ADP transport in intact mitochondria [13]. The V value at pH 6.5 and at 5 °C was between 2 and 3 nmol ADP transported per min and per mg protein. Under comparable conditions of temperature and pH, the rate of ADP transport was 5–10 times lower than that found in intact mitochondria [19].

The specificity of adenine nucleotide transport in sonic particles was studied by reverse exchange (cf. Methods). As for intact mitochondria, transport was restricted to ADP and ATP. Other nucleotides, like AMP, CDP (or GTP), UDP (or UTP), GDP (or GTP), bromoADP and diadenosinepentaphosphate were not transported at all (Table II).

ADP or ATP transport was insensitive to added carboxyatractyloside even at a concentration as high as 25 μM, which is more than 10 times higher than the concentration required to block completely ADP or ATP transport in intact mitochondria [5] (Fig. 4). In contrast, bongkrekic acid inhibited ADP or ATP transport in sonic particles with the same efficiency as in intact mitochondria [6, 20, 21]. These effects will be examined in more detail later. However, it should be noted that bongkrekic

TABLE II

SPECIFICITY OF THE ADENINE NUCLEOTIDE TRANSPORT IN SONIC PARTICLES

Transport was assayed by the reverse exchange procedure [13] with particles loaded with [^{14}C]ADP. The listed nucleotides were added at the final concentration of 100 μM. AP_5A, P^1, P^5-di(adenosine-5')-pentaphosphate; 8-BrADP = bromoADP.

Added nucleotide	Rate of exchange (percent of the maximum)
ADP	100
ATP	76
AP_5A	5
8-BrADP	0
AMP	0
GTP or GDP	0
CTP or CDP	0
UTP or UDP	0

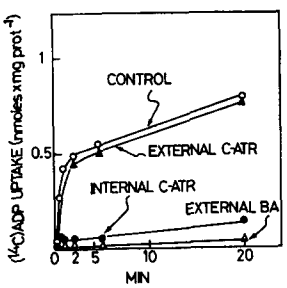

Fig. 4. Effect of atractyloside and bongkrekic acid on [^{14}C]ADP transport in sonic particles. Standard sonic particles (1 mg protein) were preincubated for 3 min at 0 °C in 1 ml of the standard incubation medium with 25 μM carboxyatractyloside or 10 μM bongkrekic acid (added carboxyatractyloside and added bongkrekic acid). The transport reaction was initiated by addition of [^{14}C]ADP (100 μM) and carried out for different periods of time at 0 °C. Sonic particles loaded with carboxyatractyloside (internal carboxyatractyloside) were prepared by sonication of mitochondria in the presence of 6 nmol of carboxyatractyloside per mg protein.

acid, when used at concentrations giving partial inhibition, did reduce the rate, but not the extent of [^{14}C]ADP or ATP uptake, a result which again favors the view that a major part of [^{14}C]ADP or ATP uptake in sonic particles is due to transport and not to binding.

Effect of FCCP on ADP and ATP transport in sonic particles

Energization of intact mitochondria brought about by addition of a respiratory substrate plus oligomycin results in an increase of the K_m value for ATP [22]. Furthermore the K_m value for ATP in energized mitochondria is markedly higher than the K_m value for ADP. In contrast, when mitochondria are deenergized by FCCP, the K_m values for ADP and ATP entry are similar [22]. Although deenergization significantly increased the rate of ATP transport at non-saturating concentrations of ATP [23], it had only a small effect at saturating concentrations of ATP, which gave a maximum rate of transport [22].

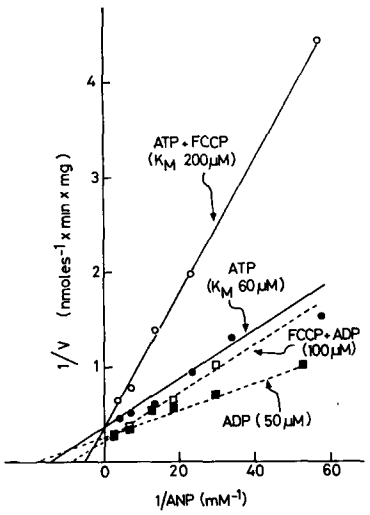

Fig. 5. Double reciprocal plot of the rate of ADP or ATP transport vs. ADP or ATP concentration in energized and deenergized sonic particles. Energized sonic particles were obtained by incubation for 3 min at 5 °C with 1 mM succinate plus oligomycin (5 μg/ml) in the standard incubation medium. Deenergized particles were obtained by incubation for 3 min at 5 °C with 1 μM FCCP. ADP and ATP transports were then assayed according to the procedure described in Methods.

The effect of energization and deenergization on the kinetic parameters of ADP and ATP transport in sonic particles is shown in Fig. 5. The K_m value for ATP uptake was 60 μM in the energized state (addition of succinate plus oligomycin); it increased to 200 μM in the deenergized state (addition of FCCP). Experiments carried out with five different preparations of sonic particles showed that the K_m values for ATP increased 2–4 times upon deenergization. In these experiments, the increase of K_m for ADP upon deenergization was between 1.4 and 2.0. It must be noted that the FCCP effect is observed essentially at high concentrations of external KCl.

There was no significant effect of the energy state on the V value for ADP or ATP uptake in sonic particles. It is noteworthy that the V value for ATP transport is lower than the V value for ADP transport, like in intact mitochondria.

FCCP, which deenergizes mitochondria, collapses the two components of the proton motive force, namely the pH gradient between the two sides of the mitochondrial membrane and also the membrane potential [24]. The effect of energization and deenergization on the pH gradient was measured in sonic particles incubated in the same medium (pH 6.5) as that used for the assay of ADP and ATP transport (see Methods). Its value was 0.9–1.5 pH units (three experiments) for the standard sonic particles prepared in the presence of $MgCl_2$ and energized by succinate plus oligomycin (internal pH < external pH); it dropped to 0.3–0.4 after addition of FCCP. It must be added that the pH gradient in energized standard particles was somewhat higher (about 2 pH units) when the pH of the incubation was raised to 7.5. Sonic particles prepared in the presence of EDTA (instead of $MgCl_2$) and energized by succinate plus oligomycin at pH 7.5 exhibited a higher pH gradient (2.5–3.0 pH units). In agreement with Rottenberg and Lee [25], the pH gradient approaches a

maximal level in EDTA sonic particles and, apparently, the membrane potential does not contribute significantly to the proton motive force in these EDTA particles. The pH gradient in the $MgCl_2$ particles used for ADP/ATP transport in the present work is smaller than in EDTA particles; therefore the contribution of the membrane potential to the proton motive force may not be negligible in $MgCl_2$ particles. Work is in progress to determine the effect of varying the membrane potential on ADP or ATP transport in $MgCl_2$ particles.

Effects of specific inhibitors on adenine nucleotide transport in sonic particles

Bongkrekic acid inhibits adenine nucleotide transport in intact mitochondria only at acidic pH [20]. Furthermore, the complete inhibition of ADP transport by bongkrekic acid in intact mitochondria requires a lag period of a few minutes [20]. The strong pH dependence and the lag period characteristic of the inhibition of ADP transport by bongkrekic acid in intact mitochondria was not observed in sonic particles (Fig. 6). The inhibitory effect of bongkrekic acid in sonic particles was strictly similar at pH 7.5 and 6.5 (Fig. 6A). Two methods have been used to determine the period of time required for complete inhibition of ADP transport by an excess of bongkrekic acid (10 μM final concentration). In the first method, bongkrekic acid was preincubated in the reaction mixture for various periods of time prior to addition of [^{14}C]ADP. The uptake of [^{14}C]ADP was terminated by centrifugation (Fig. 6B). The second method was to follow ADP transport in [^{14}C]ADP-loaded particles by back exchange. In this case, ADP transport was stopped after addition of ADP by rapid filtration on millipore (less than 2 s), with or without a prior addition of bongkrekic acid, and the radioactivity of the filtrate was determined (not shown). In both cases, the period of time required to completely block ADP transport by 10 μM bongkrekic acid at 5 °C in sonic particles was less than 5 s. The inhibition caused by bongkrekic acid in sonic particles was a mixed type inhibition, with a K_i value of 1.2 μM (Fig. 7).

As already noted (Fig. 4) carboxyatractyloside has almost no inhibitory effect on the rate of ADP transport in sonic particles. This parallels the report by Shertzer and Racker [26] who used atractyloside and showed that this inhibitor added exter-

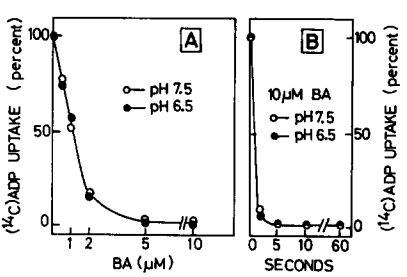

Fig. 6. (A) Effect of pH on the inhibition of ADP transport by bongkrekic acid in sonic particles. (B) Absence of lag phase for inhibition by bongkrekic acid. (A) Standard sonic particles were preincubated for 10 s at 5 °C in a series of tubes containing the standard incubation medium with increasing concentrations of bongkrekic acid (BA). Then, the transport reaction was initiated by addition of [^{14}C]ADP (100 μM) and terminated by addition of 10 μM bongkrekic acid (see Methods). (B) Standard particles were preincubated as above with 10 μM bongkrekic acid for different periods of time and then [^{14}C]ADP transport was assayed.

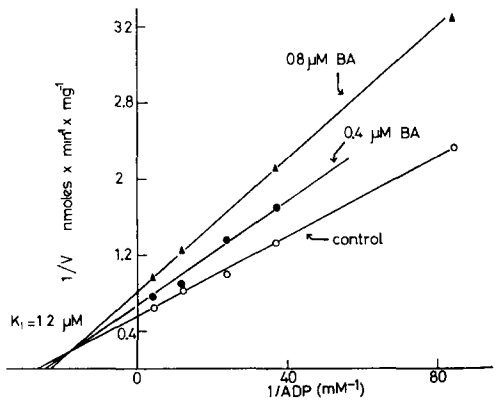

Fig. 7. Double reciprocal plot of the rate of ADP transport vs. ADP concentration at different fixed concentrations of bongkrekic acid. Standard sonic particles were preincubated for 2 min at 5 °C with bongkrekic before addition of [^{14}C]ADP. For other conditions see Methods.

nally has little effect on ATP uptake in sonic particles. However, sonic particles prepared by sonication of mitochondria in a standard medium supplemented with carboxyatractyloside (at the concentration of 6 nmol per mg protein) were no longer able to transport ADP (Fig. 4). We checked that under these conditions, carboxyatractyloside did not alter the content and distribution of the internal nucleotides. Other experiments (not shown) carried out with atractyloside led to similar results.

It is noteworthy that palmityl-CoA, a competitive inhibitor of ADP transport in mitochondria [27–29], also inhibits ADP transport, when added to sonic particles; the mixed type inhibition found with sonic particles has a K_i value of 1.6 μM, which is ten times higher than that found in intact mitochondria [29] (Fig. 8).

In agreement with Klingenberg et al. [30], a small fraction of [^{14}C]adenine

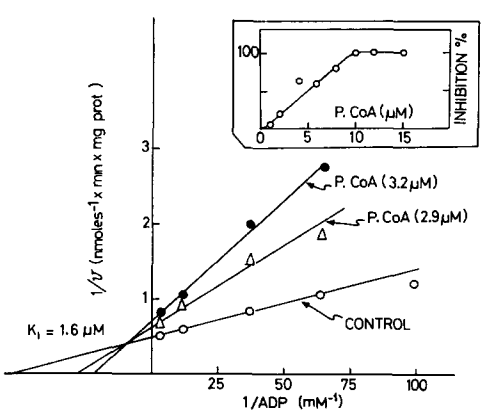

Fig. 8. Double reciprocal plot of the rate of ADP transport vs. ADP concentration at different fixed concentrations of palmityl-CoA. Standard sonic particles were preincubated for 2 min at 5 °C with palmityl-CoA before addition of [^{14}C]ADP. For other conditions see Methods. The curve in the insert illustrates the effect of increasing concentrations of palmityl-CoA on ADP transport assayed by the routine procedure, in the presence of 100 μM [^{14}C]ADP (see Methods).

nucleotides (0.15–0.20 nmol/mg protein), incorporated into sonic particles by exchange with internal adenine nucleotides under equilibrium conditions, was released upon subsequent addition of a large excess of bongkrekic acid (10 μM). Bongkrekic acid probably displaced [^{14}C]ADP and [^{14}C]ATP bound to the carrier. However, contrary to the findings of Klingenberg et al. [30], atractyloside had no effect.

Binding of [^{3}H]atractyloside and [^{3}H]bongkrekic acid to sonic particles

To elucidate the differential inhibitory effects of carboxyatractyloside (or atractyloside) and bongkrekic acid on ADP transport in sonic particles, binding assays were carried out with the radiolabeled inhibitors. [^{3}H]Bongkrekic acid was found to bind readily and with high affinity to sonic particles (Fig. 9A). The bongkrekic acid binding curve was characterized by a saturation plateau and was virtually independent of pH. The maximum amount of bongkrekic acid bound with high affinity was approx. 1 nmol/mg protein, which corresponded to about 1.3 mol of bound bongkrekic acid per mol of cytochrome *a*, based on a concentration of 0.8 nmol of cytochrome *a* per mg protein in standard sonic particles. The K_d value was about 0.4 μM. In contrast to the enhancing effect of ADP on bongkrekic acid binding in intact mitochondria, ADP added at a concentration of 50 μM to sonic particles incubated with bongkrekic acid did not affect the bongkrekic acid binding curve. Carboxyatractyloside added externally at a concentration of 10 μM did not compete with bongkrekic acid for binding to the particles. However, when carboxyatractyloside was present inside the particles (following sonication of the mitochondria with the inhibitor), it inhibited efficiently the binding of bongkrekic acid (Fig. 9B).

The [^{3}H]atractyloside binding curve did not show any clear saturation plateau (Fig. 10). However, a tentative estimation of the high affinity sites for atractyloside gave a value of 0.20–0.25 nmol of sites per mg protein ($K_d \simeq 0.2$ μM). This corresponds to about 0.30 mol of atractyloside sites per mol of cytochrome *a*, which is five times less than that found for intact mitochondria. Bongkrekic acid competed with [^{3}H]atractyloside for binding to sonic particles; ADP was much less efficient.

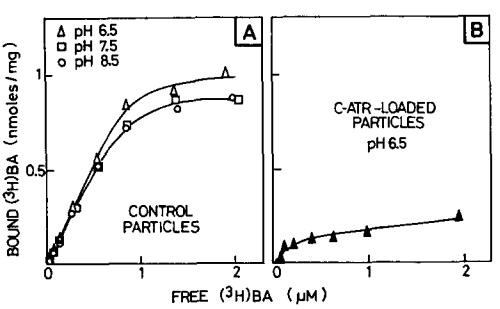

Fig. 9. Binding of [^{3}H]bongkrekic acid to standard sonic particles. (A) Effect of pH. (B) Effect of internal carboxyatractyloside. (A) Standard sonic particles (1 mg) were incubated with increasing concentrations of [^{3}H]bongkrekic acid (BA) in 1 ml of the standard incubation medium. Three parallel series of incubation, differing by the pH of the medium, were carried out. The incubation was terminated by centrifugation and the radioactivity of the pellets measured by scintillation counting. (B) Carboxyatractyloside-loaded particles were obtained by sonication of beef heart mitochondria in a standard medium supplemented with carboxyatractyloside (C-ATR) at the concentration of 6 nmol/mg protein.

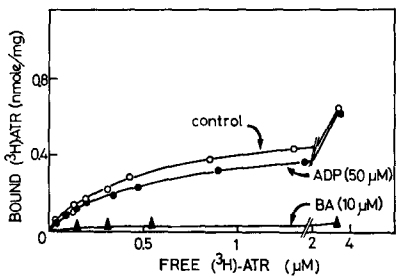

Fig. 10. Binding of [^{3}H]atractyloside to standard sonic particles. Effect of ADP and bongkrekic acid. Standard sonic particles were incubated with increasing concentrations of [^{3}H]atractyloside (ATR) in 1 ml of the standard incubation medium, pH 6.5. The incubation was terminated by centrifugation and the radioactivity of the pellets measured by scintillation counting. When present, ADP and bongkrekic acid (BA) were added together with [^{3}H]atractyloside at the concentrations of 50 and 10 μM, respectively.

DISCUSSION

This paper describes the preparation of sonic submitochondrial particles competent in adenine nucleotide transport. It was found essential to include $MgCl_2$ in the sonication medium in order to increase the amount of internal ADP and ATP and to improve the efficiency of adenine nucleotide transport. We have also found that storage of mitochondria for at least one night at −20 °C improved significantly the percentage of "inside out" particles, as assessed by the binding asymmetry of atractyloside and bongkrekic acid. Sonic particles, obtained from freshly prepared beef heart mitochondria or from mitochondria stored in liquid nitrogen, transported ADP 2–3 times faster than standard particles. The K_m value for ADP was less than 15 μM. However, these particles were still partially sensitive to external carboxyatractyloside (15–25 % inhibition of ADP transport by 25 μM carboxyatractyloside), whereas standard sonic particles are totally insensitive to external carboxyatractyloside. It is likely that particles obtained from stored mitochondria (standard sonic particles) are largely "inside out". In contrast, particles obtained from freshly prepared mitochondria are heterogeneous, and a smaller percentage are "inside out". Brief reports about sonic particles also competent in adenine nucleotide transport have appeared in literature [26, 30]. However, details of the preparation procedure were not given and furthermore there were disagreements as to the effects of atractyloside on ADP or ATP transport. The V values for ADP or ATP transport in sonic particles reported in those papers and in the present study are 5–10 times less than those found for intact mitochondria.

Standard sonic particles transport ADP and ATP by an exchange-diffusion process which has many similarities with that catalyzed by intact mitochondria. In particular the specificity of the transport for ADP and ATP and the value of the activation energy are the same in sonic particles and in intact mitochondria. On the other hand, the rate of ADP/ATP transport and the affinity for ADP or ATP are lower in sonic particles than in intact mitochondria. This suggests that the adenine nucleotide carrier has been inactivated by sonication. Other alterations found in sonic particles concern the K_i and K_d values for specific inhibitors. The K_i values for

bongkrekic acid and palmityl-CoA in sonic particles were 10 times higher than in intact mitochondria. The K_d value for bongkrekic acid in sonic particles was also 10 times higher than in intact mitochondria. In spite of the lower affinity for bongkrekic acid, the number of bongkrekic acid binding sites which reflects the density of the adenine nucleotide carrier was similar in intact mitochondria and in sonic particles (1.5–2.0 mol/mol of cytochrome *a* in mitochondria vs. 1.3 mol per mol of cytochrome *a* in sonic particles). In brief, upon sonication the number of carrier units remains practically constant, but the kinetic properties of the carrier and its affinity for substrates are significantly modified, resulting in a decrease in the efficiency of ADP/ATP transport. This may be due to the deleterious effect of sonication on some membrane parameters which probably control the conformation of transport systems, such as the direct lipid environment or the interaction with intrinsic hydrophobic proteins.

Two other major differences of adenine nucleotide transport in standard sonic particles and intact mitochondria, namely the binding asymmetry with respect to specific inhibitors and the effect of uncouplers, will now be discussed.

Binding asymmetry

In intact mitochondria atractyloside appears to bind to the ADP carrier from the outside of the inner membrane and bongkrekic acid from the inside. This is supported by the facts that in intact mitochondria, atractyloside or carboxyatractyloside binding does not depend on pH; in addition, a lag period is not required [5] which contrasts to the characteristics of bongkrekic acid binding [6]. Furthermore, bongkrekic acid accumulates in the mitochondrial matrix of intact mitochondria at acidic pH [6], which suggests that bongkrekic acid has to pass in the protonated form through the lipid core of the inner mitochondrial membrane to reach its binding site on the ADP carrier. In short in sonic particles, which have a reversed polarity with respect to that of mitochondria, bongkrekic acid binding does not require any lag and does not depend on pH.

The density of bongkrekic acid binding sites in sonic particles is similar to that found in intact mitochondria under optimum conditions of binding. In contrast, the number of atractyloside binding sites in sonic particles is less than one-fifth of that found in intact mitochondria, and one may wonder whether the atractyloside binding in sonic particles is not due to a fraction of sonic particles (one-fifth in this experiment) which are leaky to atractyloside. These leaky vesicles would also be leaky for ADP or ATP and would not obviously transport ADP or ATP by exchange-diffusion.

In good agreement with binding data, ADP transport in sonic particles is sensitive to added bongkrekic acid but not to atractyloside nor to carboxyatractyloside. However, it becomes sensitive to atractyloside or carboxyatractyloside after the vesicles have been preloaded with these inhibitors. These data taken together strongly point to the binding asymmetry of the ADP carrier with respect to atractyloside and bongkrekic acid.

The fact that internal carboxyatractyloside inhibits the binding of external bongkrekic acid (Fig. 9B) suggests that the two inhibitors either bind to two different sites of the same carrier molecule, or to the same site which is able to assume two different conformations, depending on the location of the carrier in the membrane.

In the first alternative, the transport system might be visualized as a channel, the carboxyatractyloside and bongkrekic acid sites being located on opposite sites of the channel. Thus, the interaction between the two inhibitors is indirect, i.e. the binding of carboxyatractyloside induces a change of conformation which is propagated to the bongkrekic acid site, resulting in a decrease of the affinity for bongkrekic acid and in the release of bound bongkrekic acid. The second alternative is more readily accommodated by a mobile carrier mechanism; in this case, the binding site changes its conformation when the carrier moves from the inner to the outer side of the membrane.

Effect of FCCP on adenine nucleotide transport

The rate of ATP transport in intact mitochondria at non-saturating concentration of ATP is stimulated by addition of an uncoupler, for example FCCP [23]. Assuming that the ADP/ATP exchange is electrogenic, it has been postulated that FCCP, which collapses both the pH gradient and membrane potential, abolishes the energy barrier imposed to the exchange of external ATP for internal ADP [23]. On the other hand, it has been found that in intact mitochondria FCCP markedly decreases the K_m value for ATP [22] and increases the K_m value for ADP [4], and that the maximal rate of ATP transport obtained at a saturating concentration of ATP is not significantly altered by FCCP [22]. These latter data have been interpreted to mean that the energy state of mitochondria controls the conformation of the adenine nucleotide carrier; when the energy state is low, the affinity for ATP is high and vice-versa [22].

Addition of FCCP to sonic particles does not affect the maximum rate of transport neither for ATP, nor for ADP. FCCP, however, increases the K_m values, particularly for external ATP. The dependence of the affinity for ATP on the energy state of the particles favours the view that the energy state controls the conformation of the substrate site of the adenine nucleotide carrier. The fact that the effect of FCCP on the K_m value for ATP in sonic particles is opposite to that found in intact mitochondria, is in agreement with the fact that the binding asymmetry of the ADP carrier for inhibitors in mitochondria is opposite to that observed in sonic particles. It is also in line with the reverse polarity of the membrane in sonic particles. However, in sonic particles like in intact mitochondria, the rate of ADP transport remains higher than that of ATP transport. This casts some doubt on the statement [23] that membrane potential is essentially responsible for the preferential ADP_{ex}/ATP_{in} exchange in intact mitochondria.

NOTE ADDED IN PROOF (Received March 3rd, 1977)

When the KCl concentration of the incubation medium is lowered from 125 mM to 10 mM, the V value for ADP and ATP transport is increased by two to three times and the K_m value for ADP or ATP is decreased two fold.

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