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ADENINE NUCLEOTIDE TRANSLOCATION IN YEAST MITOCHONDRIA. EFFECT OF INHIBITORS OF MITOCHONDRIAL BIOGENESIS ON THE ADP TRANSLOCASE

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SUMMARY

1. Optimal test conditions for adenine nucleotide translocation in *Candida utilis* mitochondria are a standard medium, consisting of 0.63 M mannitol, 2 mM EDTA (or ethylene glycol tetraacetic acid, EGTA), 10 mM morpholinopropane sulfonic acid (pH 6.8), and a temperature of 0 °C.

2. Adenine nucleotide translocation in *C. utilis* mitochondria is an exchange–diffusion process. The whole pool of internal adenine nucleotides is exchangeable, ADP being the most readily exchangeable nucleotide. The rate of mitochondrial ADP exchange, but not the K_m value, depends on growth conditions. At 0 °C, the rate is about 3 to 4 nmoles ADP/min per mg protein for mitochondria obtained from yeast grown in the presence of 1.5% glucose; it rises to 11.5 nmoles when glucose is replaced by 3% ethanol in the growth medium. The K_m value for ADP is 2 μM. The Q_{10} is about 2 between 0 and 20 °C. Among other exchangeable adenine nucleotides are ATP, dADP and the methylene and the hypophosphate analogues of ADP. Unlike mammalian mitochondria, *C. utilis* mitochondria are able to transport external UDP by a carboxyatractyloside-sensitive process.

3. Under conditions of oxidative phosphorylation (phosphate and substrate present in an aerated medium), added ADP is exchanged with internal ATP. A higher ATP/ADP ratio was found in the extramitochondrial space than in the intramitochondrial space. The difference between the calculated phosphate potentials in the two spaces was 0.9–1.7 kcal/mole.

4. Atractyloside, carboxyatractyloside, bongkrekic acid and palmityl-CoA inhibit mitochondrial adenine nucleotide translocation in *C. utilis* as they do in mammalian mitochondria, but 2 to 4 times less efficiently. The inhibition due to atractyloside or palmityl-CoA is competitive with respect to ADP whereas that due to bongkrekic acid and carboxyatractyloside is non-competitive. Carboxyatractyloside and atractyloside inhibitions are additive. The apparent K_d for the binding of [^{35}S]-carboxyatractyloside and [^{14}C]bongkrekic acid is 10–15 nM and the concentration of sites 0.4–0.6 nmole/mg protein in both cases. [^{35}S]Carboxyatractyloside binding is competitively displaced by atractyloside and *vice versa*.

Abbreviations: APP, adenosine 5′-hypophosphate; APCH$_2$P, adenosine 5′-methylene diphosphate; MOPS, morpholinopropane sulfonic acid; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; GUM, carboxyatractyloside (used interchangeably with gummiferin).

5. Binding of [^{14}C]ADP has been carried out with mitochondria depleted of their endogenous adenine nucleotides by incubation with phosphate and Mg^{2+} at 20 °C. The amount of bound [^{14}C]ADP which is atractyloside removable is 0.08–0.16 nmole/mg protein.

6. The rate of ADP transport is quite different in mitochondria isolated from *C. utilis*, according to whether it is grown on glucose, or on ethanol or in the presence of chloramphenicol; for instance, it decreases by 10 times when 3% ethanol in the growth medium is replaced by 10% glucose and by 5 times when chloramphenicol is added to the medium. These variations are accompanied by parallel variations in cytochrome aa_3. The number of atractyloside-sensitive ADP binding sites is not modified by the above conditions of culture, nor the number of [^{35}S]carboxyatractyloside binding sites. The affinity for ADP is apparently not significantly modified, nor the size of the endogenous adenine nucleotide pool. In contrast to glucose repression or chloramphenicol inhibition, semi-anaerobiosis in *C. utilis* lowers significantly the mitochondrial binding capacity for carboxyatractyloside. Strict anaerobiosis in *S. cerevisiae* results in a practical loss of the cytochrome oxidase activity, and also of the carboxyatractyloside and ADP binding capacity. Transition from anaerobiosis to aerobiosis restores the cytochrome oxidase activity and the ADP and carboxyatractyloside binding capacities.

INTRODUCTION

Yeast cells afford the possibility of phenotypic variations which can be easily obtained either by environmental changes or by cytoplasmic or nuclear mutations. Some of these variations are of potential value to study the molecular mechanism of mitochondrial processes, such as oxidative phosphorylation or ion transport. With this line of thinking and in view of our current interest in the mechanism of adenine nucleotide translocation, we have investigated the effect of various inhibitors of mitochondrial protein synthesis on the activity of the ADP translocator and on the binding of specific inhibitors to mitochondrial membranes using mitochondria from *Candida utilis* and, in addition, those of *Saccharomyces cerevisiae*. Results concerning this study are reported in this paper, as well as an account of the properties of adenine nucleotide translocation under optimal test conditions.

MATERIAL AND METHODS

Chemicals

Atractyloside and carboxyatractyloside (gummiferin) were extracted from the rhizomes of the thistle *Atractylis gummifera* and purified as described in previous reports[1,2]. ^{35}S-labelled atractyloside and ^{35}S-labelled carboxyatractyloside were prepared in the same way from thistles that had been grown in the presence of [^{35}S]-sulfate. Bongkrekic acid (Professor Berends) was obtained through the generosity of Professor Lederer. Using the same method as for unlabelled bongkrekic acid[3,4], we isolated [^{14}C]bongkrekic acid from cultures of *Pseudomonas cocovenenans* incubated for 4 days at 30 °C and a relative humidity of 70% on defatted coconut supplemented with [^{14}C]glycerol or [^{14}C]glucose. Palmityl-CoA was synthesized either from the corresponding anhydride[5] or chloride derivative[6].

[^{14}C]ADP was obtained from Schwarz Bioresearch and the phosphonic analogue of ADP (adenosine 5'-methylene diphosphonate, $APCH_2P$) from Miles. [^{14}C]-UDP, [^{14}C]CDP and [^{14}C]GDP (Schwarz Bioresearch) were prepared free from adenine nucleotide contaminant as previously described[7].

Yeast culture

Two strains of yeasts were used: *Candida utilis* (CBS 1516) and *Saccharomyces cerevisiae* (IL8-8C). Both of them were grown at 28 °C in 14-l fermentor jars (New Brunswick Scientific apparatus) containing 10 l of medium made of 2% bacto peptone (Difco), 1% yeast extract (Difco) and 1.5% glucose. In a few specific instances, the glucose concentration was increased to 10% or glucose was replaced by 3% ethanol. The culture was started by addition of 150 ml of a preculture grown for 24 h in the same medium. Aerobiosis was achieved by air bubbling at the rate of 1 l/min per l of medium and vigorous stirring (150 rev./min). Cells were harvested after 22 h (early stationary phase) by centrifugation in a Sharples centrifuge and washed 3 times with ice-cold water. The yield was 400–500 g (wet weight) of cells per 10 l.

S. cerevisiae was used in experiments requiring anaerobic growth. In this case, the growth medium was supplemented with 5 ml Tween 80 and 20 mg ergosterol per l; it was freed of oxygen by bubbling with a stream of nitrogen purified by passage through a suspension of yeast cells as described by Criddle and Schatz[8] to remove the last traces of oxygen from nitrogen. The anaerobic cells were harvested in a precooled Sharples centrifuge flushed with nitrogen. They were subsequently washed with cold nitrogen-saturated water.

Cytochrome aa_3 concentration in cells was estimated by recording the difference in absorbance of the yeast suspension at 605 nm and 630 nm in a dual wavelength Aminco–Chance spectrophotometer. Full oxidation was achieved by addition of 5 μl of 10% H_2O_2 to 3 ml of the yeast suspension and full reduction by addition of dithionite. $\Delta\varepsilon$ (605—630 nm) was taken to be 24 $mM^{-1} \cdot cm^{-1}$ (ref. 9).

Preparation of mitochondria

Yeast cells were disrupted in an Eppenbach micromill (Gifford-Wood) essentially as described by Balcavage and Mattoon[10] with the following minor modifications. The homogenization medium was made up of 0.63 M mannitol, 0.4% bovine serum albumin (Fraction V Sigma), 2 mM EDTA, and 10 mM morpholinopropane sulfonic acid (MOPS) buffer, pH 6.8. Before being ground, yeast cells were washed once with the homogenization medium, and then mixed with an equal volume of medium and 2 vol. of 0.17–0.18 mm glass beads (Braun). The degree of coupling of these mitochondria was assessed by measuring the respiratory control with an oxygraph equipped with a Clark electrode. Respiratory control indices of 3 or higher were routinely obtained using malate *plus* pyruvate as oxidizable substrates. Mitochondrial preparations which did not satisfy this criterion were discarded. Cytochrome oxidase activity as a marker of the inner mitochondrial membrane was assayed according to Appelmans *et al.*[11].

Determination of the matrix space

C. utilis mitochondria were incubated with a medium containing [^{14}C]mannitol and then centrifuged in tared polypropylene centrifuge tubes for 5 min at 20000 $\times g$ at

0 °C. The supernatant fluid was decanted and the interior of the tubes carefully blotted with filter paper before weighing. For dry weight measurement, the tubes containing the mitochondrial pellets were dried overnight at 80 °C, cooled and reweighed. The dry pellets were then digested with 1% sodium cholate for counting the [^{14}C]mannitol radioactivity. The volume of the matrix space (mannitol-impermeable space) was taken as the difference between the total water space as determined by gravimetry and the mannitol space as determined with [^{14}C]mannitol.

Measurement of adenine nucleotide translocation

In most experiments, the adenine nucleotide translocation was measured by the back exchange procedure[7,12]. Mitochondria suspended at a concentration of about 15 mg/ml of 0.63 M mannitol, 0.2% bovine serum albumin, 10 mM MOPS and 0.1 mM EDTA, pH 6.8, were first loaded with [^{14}C]adenine nucleotide by incubation with 20 μM [^{14}C]ADP for 45 min at 0 °C, washed twice with the suspension medium to remove [^{14}C]adenine nucleotides external to the matrix space and finally resuspended in the same medium to a concentration of about 30 mg of protein/ml. They contained 3–5 nmoles of adenine nucleotides per mg protein. The standard medium used for translocation assays was made up of 0.63 M mannitol, 2 mM EDTA, 10 mM MOPS, pH 6.8. The [^{14}C]ADP-loaded mitochondria were added to this medium at about 1 mg/ml and preincubated under aerobiosis for 30 s at 0 °C, except in experiments where the inhibition by bongkrekic acid was studied; in this case the preincubation was carried out at 25 °C. The exchange was initiated by injection of unlabelled ADP and stopped after incubation periods from 10 s to 10 min by rapid filtration (less than 2 s) through a 0.45-μm millipore filter HAWP inserted in a filter holder fitted to a syringe. Spontaneous leakage was measured by the same procedure in the absence of external ADP. The radioactivity of the filtrate was estimated by liquid scintillation counting; the scintillation fluor used contained 100 g of naphthalene, 6 g of 2,5-diphenyloxazole and 300 mg of 2,2′-*p*-phenylene-bis-(4-methyl-5-phenyloxazole) per l of 1,4-dioxane. The percentage of internal adenine nucleotide exchanged with added ADP was usually calculated as $100\times(a-b)/c$, where a is the radioactivity of the filtrate obtained in the presence of added ADP, b that in the absence of added ADP (spontaneous leakage) and c the initial radioactivity of the labelled mitochondria. In kinetic experiments, account was taken of the fact that ADP and ATP are exchanged much more rapidly than AMP; the percentage of exchange of internal adenine nucleotides with external ADP was therefore calculated as $100\times(a-b)/c\cdot r$, where r is the ratio of internal ADP *plus* ATP to the sum of the internal AMP, ADP and ATP. Internal [^{14}C]ATP, [^{14}C]ADP and [^{14}C]AMP in neutralized perchloric acid extracts of labelled mitochondria were assayed by enzymatic methods[13] and also separated by paper chromatography[14]. $HClO_4$ extracts were obtained by adding to an aliquot of the mitochondrial suspension (30 mg/ml) $HClO_4$ to a final concentration of 2%. After 1 h at 0 °C, denatured proteins were discarded by centrifugation, the supernatant fluid was neutralized with KOH and $KClO_4$ removed by centrifugation at 0 °C. The r ratio was calculated from the amount of mitochondrial AMP, ADP and ATP determined enzymatically or from the respective radioactivity of [^{14}C]AMP, [^{14}C]ADP and [^{14}C]ATP assuming complete enzymatic equilibration between the [^{14}C]adenine nucleotides in labelled mitochondria. Both methods yielded similar values of r. In a few experiments where incubation lasted for several minutes or when

added nucleotides other than adenine nucleotides were used, the translocation was assayed by direct uptake of [^{14}C]nucleotides followed by rapid centrifugation. The mitochondrial pellets were dissolved in 1 ml of formamide at 180 °C and their radioactivity determined by liquid scintillation.

Measurement of phosphate potential

C. utilis mitochondria were incubated in aerobiosis in a medium containing [^{32}P]phosphate, ADP and an oxidizable substrate. Three parallel series of incubation were carried out. Incubation in the first series of tubes was ended by addition of $HClO_4$ (0.25 M final concentration) and in the second series of tubes by filtration through millipore filter. The difference between the respective amounts of AMP, ADP and ATP in the total $HClO_4$ extracts (Series 1) and in the filtrates (Series 2) gives the amount of each nucleotide present in aerobiosis within the mitochondrial particles. Incubation was stopped by centrifugation in the third series of tubes (in duplicate). Some tubes were used for gravimetric determination of water in the pellets and estimation of the extra matrix space, assuming a matrix volume of 0.9 μl/mg protein (see first section of Results). In others, the pellets were extracted with 3 ml of 2.5 M $HClO_4$. Adenine nucleotides were assayed enzymatically in an aliquot of this $HClO_4$ extract. Another aliquot was chromatographed to separate $^{32}P_i$ (ref. 15). The amount of P_i in the pellet was calculated from the total radioactivity of $^{32}P_i$ in the pellet and the specific radioactivity of $^{32}P_i$ in the medium assuming rapid equilibration of P_i between extramitochondrial and intramitochondrial spaces. The amount of P_i in the matrix space was obtained after correction for the P_i content of the extra matrix space (see above). It was the sum of the original P_i in aerobic mitochondria and of the P_i released from ATP or ADP by dephosphorylation in the anaerobic mitochondrial pellet. P_i released by anaerobiosis was easily calculated as the difference between the total phosphate in the mitochondrial adenine nucleotides under aerobic conditions (Series 1–2) and under anaerobic conditions (Series 3).

RESULTS

(1) Optimal test conditions for measurement of the transmembrane adenine nucleotide exchange

To assay accurately adenine nucleotide translocation in mitochondria, the concentration of internal adenine nucleotides must remain stable. The effect of several factors, namely osmolarity, pH, temperature and ion specificity, on the stability of the internal adenine nucleotide pool was therefore investigated.

(a) Osmolarity. As found by Onishi *et al.*[16] with *Saccharomyces carlsbergensis* and Balcavage and Mattoon[10] with *S. cerevisiae*, a high osmolarity of the incubation medium is required for mitochondria to exhibit satisfactory respiratory control or high P/O ratios. In agreement with these data, Kolarov *et al.*[17] have reported that *S. cerevisiae* mitochondria rapidly swell when the suspension medium is less than 0.6 osM. Data in Fig. 1 illustrate the effect of osmolarity of the incubation medium on the mitochondrial matrix and on adenine nucleotide translocation in *C. utilis* mitochondria. They show a linear relationship between the matrix space and the reciprocal of the osmolarity of the incubation medium, at least between 0.63 and 0.23 M man-

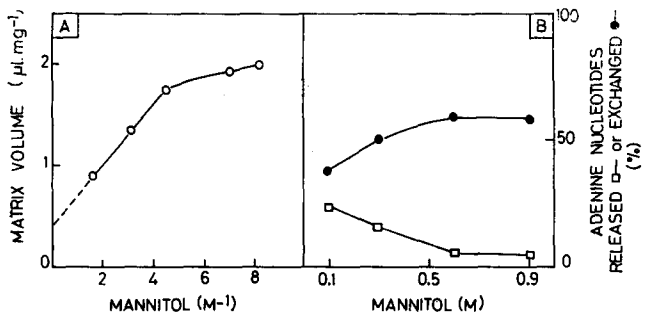

Fig. 1. Effect of osmolarity of the medium on the matrix volume of mitochondria, on the ADP transport and the on leakage of internal adenine nucleotides. (A) *C. utilis* mitochondria (11.8 mg protein) were incubated for 2 min at 0 °C in 3.1 ml of 2 mM EGTA, 10 mM MOPS, pH 6.8, and [^{14}C]mannitol at the indicated concentrations. The volume of the matrix space was determined as indicated in Methods. (B) [^{14}C]ADP-loaded *C. utilis* mitochondria (1.9 mg protein) were incubated at 0 °C in 1.1 ml of the same medium as in A, in the absence of in the presence of 360 μM ADP. The incubation was stopped after 10 min at 0 °C by rapid centrifugation. The percentage of [^{14}C]adenine nucleotides spontaneously released in the absence of added ADP or exchanged with the added ADP was estimated in the supernatant fluid by liquid scintillation counting.

nitol (Fig. 1A), indicating that, in a given range of mannitol concentrations, *C. utilis* mitochondria behave as true osmometers. In the routine assay conditions of ADP translocation where the mannitol concentration was 0.63 M, the volume of the matrix space was of the order of 0.9 μl/mg protein. The adenine nucleotide exchange (after correction for spontaneous leakage) was about 60% lower in 0.10 M mannitol than in 0.60 M mannitol; it was maximal from 0.60 to 0.90 M mannitol (Fig. 1B). The decrease in exchange below 0.60 M mannitol was apparently correlated with a leakage of internal adenine nucleotides (Fig. 1B), which may depend on the increase of the matrix space (Fig. 1A), and the subsequent loss of the structural integrity of the inner mitochondrial membrane.

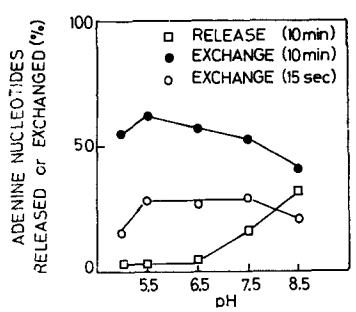

Fig. 2. Effect of pH on the ADP exchange and on the leakage of internal adenine nucleotides. [^{14}C]ADP-loaded *C. utilis* mitochondria (1.8 mg protein) were incubated at 0 °C in 3.2 ml of 0.63 M mannitol, 2 mM EGTA, 5 mM 2-(*n*-morpholino)ethane sulfonic acid and 5 mM Tris–HCl at the indicated pH, in the absence of ADP (leakage) or in the presence of ADP (exchange). Incubation was stopped by filtration on millipore filter after 15 s or 10 min. The percentage of [^{14}C]adenine nucleotides released or exchanged was calculated from the radioactivity of the supernatant fluid.

(*b*) *pH effect.* As shown in Fig. 2, the exchange (after correction for spontaneous leakage) was virtually the same from pH 5.0 to pH 8.5 when measured over a short period of time (15 s); however, it was significantly decreased above pH 6.5 for longer periods of incubation (10 min). Here again, the decrease of exchange was correlated with an increased leakage of internal nucleotides which could reflect a damage of the mitochondrial membrane.

(*c*) *Temperature and ion effect.* In suspensions of freshly prepared *C. utilis* mitochondria, 5 to 10% of the total of adenine nucleotide was found in the supernatant fluid obtained after centrifugation. The presence of external adenine nucleotides in these suspensions is explained by the membrane alteration of a small fraction (5 to 10%) of mitochondria. The pool of internal adenine nucleotides remained virtually stable when mitochondria were allowed to stand at 0 °C in their standard suspension medium for periods of time not exceeding 2 to 3 h. At 20 °C a significant leakage occurred; for instance, a 1-h incubation at 20 °C resulted in a release of 15 to 25% of the internal adenine nucleotides. As shown in Fig. 3A, the leakage at 20 °C was enhanced by phosphate and partly prevented by Mg^{2+}. When added simultaneously with phosphate, Mg^{2+} unexpectedly increased the phosphate-induced release of adenine nucleotides. Very high concentrations (60 μM) of two inhibitors of the adenine nucleotide translocation, namely atractyloside or bongkrekic acid, partially prevented the leakage (Fig. 3B). A similar effect of bongkrekic acid in mammalian mitochondria has been reported[18].

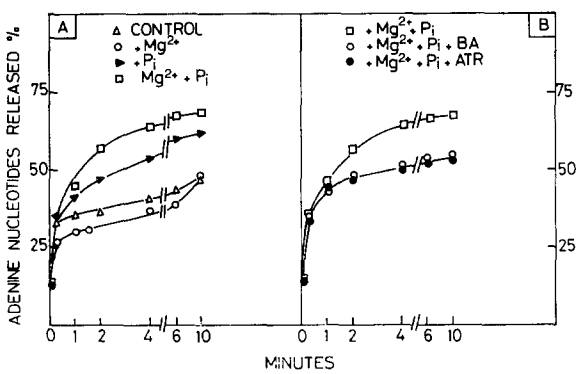

Fig. 3. Leakage of internal adenine nucleotides at 20 °C. Effect of phosphate and Mg^{2+}. Antagonistic effect of atractyloside and bongkrekic acid. [^{14}C]ADP-loaded *C. utilis* mitochondria (2.7 mg protein) were incubated at 20 °C in 1.1 ml of 0.63 M mannitol, 2 mM EDTA and 10 mM MOPS, pH 6.8. Where present, P_i was 6 mM, $MgCl_2$ 6 mM and either bongkrekic acid (BA) or atractyloside (ATR) 60 μM. Incubation was stopped by centrifugation at the indicated time.

A number of cations were tested at 0 °C on both the exchange and the leakage of internal adenine nucleotides. Control assays without added cation included EDTA or ethylene glycol tetraacetic acid (EGTA) as metal-complexing agents. EGTA and EDTA gave similar results. At a final concentration of 3.3 mM the cations Mn^{2+} and Fe^{2+} did not significantly modify exchange or leakage over an incubation period of 10 min. Cu^{2+} was a strong inhibitor of both processes. Mg^{2+} and Ca^{2+} decreased the leakage by 2 to 3% and increased the exchange by about 10%. However, because

TABLE I

EFFECT OF THE REPLACEMENT OF MANNITOL BY KCl ON THE ADP TRANSLOCATION AND ON ITS INHIBITION BY ATRACTYLOSIDE

[^{14}C]ADP-loaded *C. utilis* mitochondria (4.9 mg protein) were preincubated for 30 s at 0 °C in 2.1 ml of 2 mM EDTA, 10 mM MOPS, pH 6.8, and mannitol or KCl as indicated in the table. Where present, atractyloside was 6 μM. Exchange was initiated by 200 μM ADP and stopped after 30 s by millipore filtration (*cf.* Methods).

Medium	*% of total internal adenine nucleotides exchanged*		*Inhibition by atractyloside* (%)
	Control	*With atractyloside*	
0.63 M mannitol	55	33	40
0.31 M KCl	61	48	21
0.59 M mannitol + 0.02 M KCl	60	44	27

of possible secondary effects, especially the activation of adenylate kinase by Mg^{2+}, these cations were not used in the routine assays of ADP translocation.

Replacing mannitol by KCl and keeping constant the osmolarity of the medium led to a small but significant increase of the ADP exchange (Table I), in agreement with data reported by Meisner[19] concerning mammalian mitochondria. However, in spite of an increased rate of exchange in a KCl medium, the inhibitory effect of atractyloside was lowered.

This series of assays led us to choose as routine test conditions to study ADP translocation in yeast mitochondria a standard medium made up of 0.63 M mannitol, 2 mM EDTA (or EGTA), 10 mM MOPS, pH 6.8, and a temperature of 0 °C. Mitochondria were used for experiments for up to 3 h after preparation.

(2) Specificity

A number of adenine nucleotides (Table II) have been tested for their specificity in the translocation process, including $APCH_2P$ and adenosine 5'-hypophosphate (APP), two ADP analogues which are transported in rat liver mitochondria, but are not phosphorylated[20,21]. ADP was the most rapidly exchangeable adenine nucleotide. The others were translocated in the following order of efficiency: ATP, dADP, $APCH_2P$, APP and AMP. All exchanges were inhibited to a similar extent by carboxyatractyloside (gummiferin), a specific non-competitive inhibitor of ADP translocation in mitochondria[22]. Cyclic AMP was totally ineffective. A slow exchange with AMP has also been reported in *S. carlsbergensis* by Onishi *et al.*[23]; it may be due to some ADP arising from transphosphorylation between the added AMP and ATP released by the mitochondria.

C. utilis mitochondria incubated with [^{14}C]CDP or [^{14}C] GDP incorporated a small amount of radioactivity, but this uptake showed little sensitivity to carboxyatractyloside (Table III) a result pointing to a non-specific uptake in agreement with data obtained with mammalian mitochondria[7,12,24,25]. Unexpectedly, however,

TABLE II

SPECIFICITY OF ADENINE NUCLEOTIDE TRANSPORT IN *CANDIDA UTILIS* MITOCHONDRIA. EFFECT OF CARBOXYATRACTYLOSIDE

[^{14}C]ADP-loaded mitochondria (6.2 mg protein) were preincubated for 30 s at 0 °C in 2.5 ml of standard medium. Exchange was initiated by 360 μM nucleotide and stopped after 30 s by millipore filtration. Where present, carboxyatractyloside was 6.2 μM.

Added nucleotide	*% of total adenine nucleotides exchanged (ADP control = 100)*		*Inhibition by carboxyatractyloside (%)*
	Control	*With carboxyatractyloside*	
ADP	100	50	50
ATP	61	40	34
dADP	50	25	50
$APCH_2P$	29	11.5	63
APP	20	7.5	62
AMP	10	5	50
Cyclic AMP	0	0	

TABLE III

INCORPORATION OF EXTERNAL ^{14}C-LABELLED NUCLEOTIDES. EFFECT OF CARBOXYATRACTYLOSIDE

The incubation medium for *C. utilis* mitochondria (5.6 mg protein) was the standard medium (1.1 ml). In the case of rat liver mitochondria (6 mg protein), it was made of 0.11 M KCl, 0.02 M Tris–HCl, pH 7.4, and 2 mM EDTA. Where present, carboxyatractyloside was 10 μM. In both cases the incubation was carried out at 0 °C for 2 min with 200 μM of [^{14}C]ADP, [^{14}C]UDP, [^{14}C]CDP and [^{14}C]GDP, and stopped by centrifugation. Corrections were made for the extra-matrix space determined in parallel assays with [^{14}C]mannitol.

Added nucleotides	*Nucleotides incorporated in matrix (nmoles)*			
	C. utilis		*Rat liver*	
	Control	*+ Carboxyatractyloside*	*Control*	*+ Carboxyatractyloside*
[^{14}C]ADP	7.8	3.1	12.9	1.5
[^{14}C]UDP	1.2	0.2	0.4	0.4
[^{14}C]CDP	< 0.1	< 0.1	0.2	0.2
[^{14}C]GDP	0.5	0.3	1.1	1.2

[^{14}C]UDP incorporation was inhibited by carboxyatractyloside (Table III), suggesting that the ADP carrier in *C. utilis* mitochondria accepts UDP as a substrate in contrast to mammalian mitochondria. Using [^{14}C]ADP-loaded mitochondria, it was found that the UDP uptake was accompanied by an efflux of [^{14}C]adenine nucleotides.

(3) Kinetics of the ADP translocation

To follow the kinetics of the ADP translocation, an aliquot of a concentrated suspension of [^{14}C]ADP-loaded mitochondria was mixed with the aerated standard medium and let to stand at 0 °C for 30 s. The exchange was initiated by addition of a saturating concentration of unlabelled ADP. After correction for spontaneous leakage, it was found that the totality of the intramitochondrial adenine nucleotide pool is exchangeable (Fig. 4A). The kinetics of ADP exchange in *C. utilis* mitochondria at 0 °C was a two-step process with an initial rapid first-order exchange ($k_1 = 2.8$ min^{-1}) lasting for about 30 s, followed by a much slower exchange which nearly reached its completion in 20 min. Only a fraction, 40%, of the internal adenine nucleotides was rapidly exchangeable; it corresponded to the sum of ADP and ATP. In agreement with the observation that AMP is very slowly exchanged, it is inferred that the rapid initial phase involves essentially the exchange of the added ADP with internal ADP *plus* ATP. Similar data have been reported by Pfaff *et al.*[26] in the case of rat liver mitochondria.

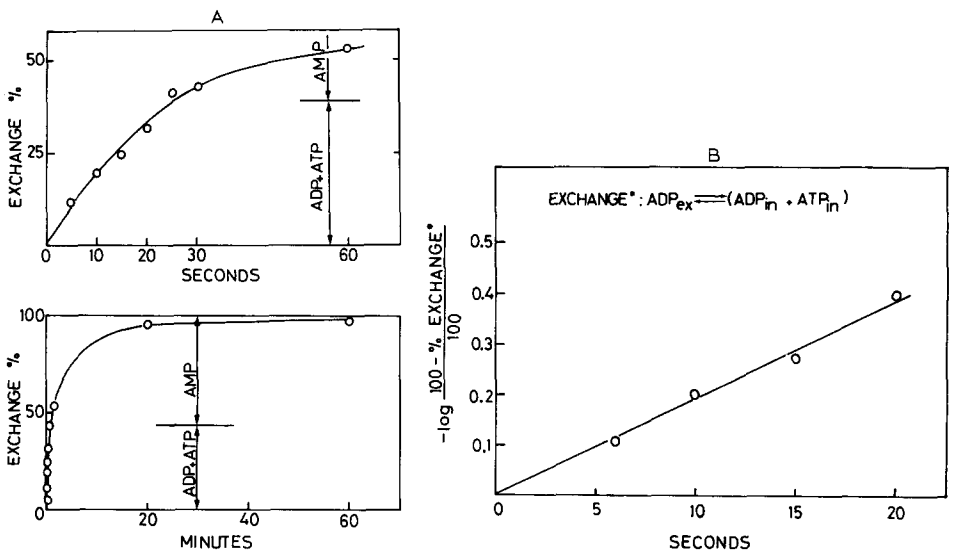

Fig. 4. (A) Kinetics of the ADP translocation in *C. utilis* mitochondria. [^{14}C]ADP-loaded mitochondria (3.3 mg protein) were preincubated for 30 s at 0 °C in 3 ml of the standard incubation medium as described in Methods. Exchange was initiated by 530 μM ADP and stopped by millipore filtration. (B) First-order reaction plot of the time variation of the ADP translocation. Data are taken from A. In this case they refer to the exchange of the added ADP with internal ADP *plus* ATP.

From the rate constant k_1, the initial rate of exchange of added ADP with internal ADP *plus* ATP at 0 °C was approximated to be 3.3 μmoles·min^{-1}·g^{-1} (Fig. 4B). This value is typical of mitochondria prepared from *C. utilis* grown in the presence of 1.5% glucose. As shown thereafter (Table VII), when glucose in the growth medium was replaced by 3% ethanol, the mitochondrial transport of ADP was much faster and reached values as high as 11.5 μmoles·min^{-1}·g^{-1}. The K_m for the transport of ADP was about 2 μM (Fig. 5).

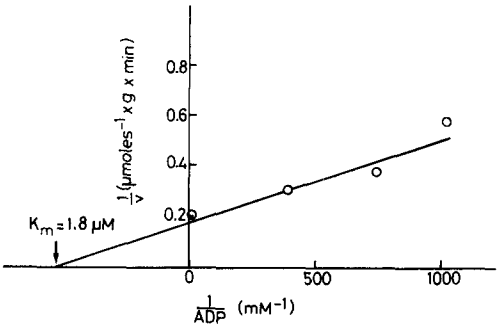

Fig. 5. Effect of ADP concentration on ADP translocation. [^{14}C]ADP-loaded *C. utilis* mitochondria (0.9 mg protein) in 2.1 ml of standard medium were incubated with different concentrations of external ADP at 0 °C for 15 s. Incubation was stopped by millipore filtration.

The effect of temperature on the rate of the ADP translocation has been tested between 0 and 20 °C. In contrast to rat liver mitochondria which exhibited a biphasic Arrhenius plot with a Q_{10} as high as 10 below 7 °C[6,26], the values of Q_{10} with *C. utilis* mitochondria were uniform in the range of temperatures tested and roughly equal to 2. In some experiments, unexpected lower values of Q_{10} (1.5 to 1.8) were found below 6 °C. This result confirms that obtained by Onishi *et al.*[23] with *S. carlsbergensis.*

Addition of phosphate to *C. utilis* mitochondria in aerobiosis with or without an oxidizable substrate led to a substantial increase of the level of internal ATP (10 to 20% in 10 min), even at 0 °C, as observed in rat liver mitochondria[27]. This increase was obviously related to the oxidation of added or endogenous substrates and to the linked phosphorylation. Arsenate brought about opposite effects. These observations provided a means of testing the effect of the mitochondrial ADP and ATP concentration on the rate of transport of external ADP. For this purpose, $APCH_2P$, an analogue of ADP which is a substrate for the ADP carrier but not a phosphate acceptor in oxidative phosphorylation[20], was chosen as external adenine nucleotide. As shown in

TABLE IV

EFFECT OF ADDED PHOSPHATE ON THE ADENINE NUCLEOTIDE EXCHANGE

[^{14}C]ADP-loaded *C. utilis* mitochondria (2.9 mg protein) were incubated in 1.1 ml of standard medium with 455 μM $APCH_2P$ for 2 min at 0 °C. Where present, phosphate and arsenate were 10 mM and succinate 10 mM. Incubation was stopped by centrifugation. A parallel experiment was carried out under similar conditions as above (except that $APCH_2P$ was absent); in this case incubation was stopped by addition of 1 ml of 2.5 M $HClO_4$. Adenine nucleotides were assayed in the $HClO_4$ extracts.

Additions	*% of internal adenine nucleotides exchanged*	$\frac{\text{Internal ATP}}{\text{Internal ADP}}$
None	27	0.7
Phosphate	40	1.0
Phosphate + succinate	36	1.1
Arsenate	20	0.4

TABLE V

FREE ENERGY OF HYDROLYSIS OF EXTRAMITOCHONDRIAL AND INTRAMITOCHONDRIAL ATP IN *C. UTILIS* MITOCHONDRIA

C. utilis mitochondria (11 mg protein) were incubated for different periods of time at 0 °C in aerobiosis in 3 ml of 0.6 M mannitol, 10 mM KCl, 10 mM Tris–maleate, 2 mM EDTA, 7.5 mM [^{32}P]phosphate, 10 μM ADP and 5 mM ethanol, final pH 6.8. Three parallel series of incubation were carried out as detailed in Methods, for the determination of internal and external ADP, ATP and phosphate.

Compartment		*2 min*	*5 min*	*10 min*
Intramitochondrial	[ATP]/[ADP]	0.92	1.36	1.62
	[ATP]/([ADP] [P_i]) (M^{-1})	114	168	200
	RT ln [ATP]/([ADP] [P_i])	2.6	2.8	2.9
	ΔG_{in}'*	9.4	9.6	9.7
Extramitochondrial	[ATP]/[ADP]	2.54	4.10	4.46
	[ATP]/([ADP] [P_i]) (M^{-1})	342	550	588
	RT ln [ATP]/([ADP] [P_i])	3.2	3.4	3.5
	ΔG_{ex}'*	11.1	11.3	11.4

* $\Delta G' = \Delta G_0' - RT$ ln [ATP]/([ADP][P_i])
$\Delta G'_0$ was taken equal to −6.8 kcal for the intramitochondrial ATP (Mg^{2+} present) and to −7.9 kcal for the extramitochondrial ATP (EDTA present)[28].

Table IV, the exchange of $APCH_2P$ with internal adenine nucleotides was markedly enhanced by phosphate alone or by phosphate *plus* succinate but lowered by arsenate. The increase in the rate of exchange was accompanied by a parallel increase in the ratio ATP_{in}/ADP_{in}. Similar data have been obtained with rat liver mitochondria[27]; they are in line with the finding that external ADP (or $APCH_2P$) is exchanged with internal ATP in preference to internal ADP[12]. In the same manner, it was found that during incubation of *C. utilis* mitochondria with ethanol as an oxidizable substrate and ADP the ATP/ADP ratio is 3–4 times higher in extramitochondrial space than in matrix space (Table V). The free energy of hydrolysis of internal and external ATP has been calculated by taking the standard free energy of ATP hydrolysis $\Delta G_0'$ (ref. 28) equal to −7.9 for the external ATP (Mg^{2+} absent) and to −6.8 for the internal ATP (Mg^{2+} present). The free energy of hydrolysis of internal ATP was found to be about 1.7 kcal lower than that of the external ATP, assuming similar activity coefficients for nucleotides and phosphate inside and outside mitochondria. Values between 0.9 and 1.7 kcal were found in four experiments. Differences of the same order[27] or higher[29,30] have been reported in the case of mammalian mitochondria.

(4) Inhibitors of ADP translocation in C. utilis mitochondria

So far, four compounds, atractyloside[31], carboxyatractyloside[1,22], bongkrekic acid[32–34] and long-chain acyl-CoA derivatives[35–37] have been characterized as potent (effective at less than 1 μM) and rather selective inhibitors of the ADP translocation in mammalian mitochondria. These inhibitors are also active in yeast mitochondria, but 2 to 4 times less than in rat liver mitochondria. In spite of their lower efficiency which may be due to some alteration of the ADP carrier resulting from the prepara-

tion step of yeast mitochondria, all four inhibitors displayed inhibitory properties similar to those found with rat liver mitochondria.

Atractyloside behaved as a competitive inhibitor of the ADP or ATP transport with respect to ADP, and bongkrekic acid behaved as a non-competitive inhibitor (Fig. 6). In the case of atractyloside, the departure of plots from linearity was probably due to the high affinity of atractyloside for mitochondrial membranes and to the large variation of the free atractyloside concentration during titration by atractyloside in the presence of ADP, a situation which generally occurs for tightly bound competitive inhibitors[38]. Bongkrekic acid was more effective after a short preincubation (30 s) at 25 °C than at 0 °C, in agreement with data reported by Kemp *et al.*[34] for mammalian mitochondria.

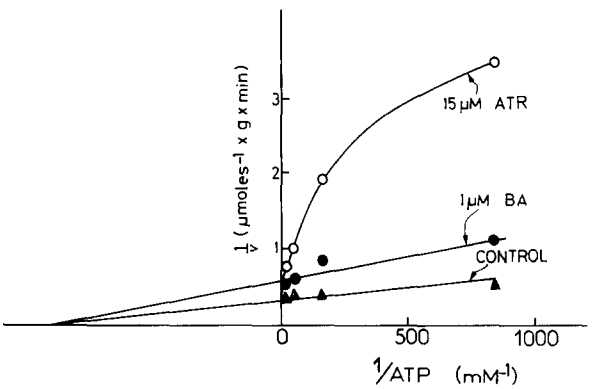

Fig. 6. Inhibition of ATP translocation by atractyloside and bongkrekic acid. [^{14}C]ADP-loaded *C. utilis* mitochondria (0.8 mg protein) were preincubated for 30 s at 0 °C in 2.4 ml of standard medium without (control) or with atractyloside (ATR). In the case of bongkrekic acid (BA), preincubation was carried out at 23 °C for 1 min. After cooling, exchange was initiated at 0 °C with ATP and stopped after 20 s by millipore filtration.

In contrast to rat liver mitochondria where the maximum of the inhibition at a given concentration of gummiferin is reached in less than 5 s at 0 °C (unpublished data), the inhibitory effect of carboxyatractyloside in *C. utilis* mitochondria was barely complete after 1 min of preincubation at 0 °C. This may be related to the rather low kinetics of carboxyatractyloside binding to *C. utilis* mitochondria, as shown thereafter. The time required to stop the ADP transport can be shorter by increasing the concentration of carboxyatractyloside; for instance, full inhibition of ADP transport at 0 °C is obtained in less than 20 s by 20 μM carboxyatractyloside. The inhibitory effect of carboxyatractyloside was not relieved by high concentrations of ADP, which points to a non-competitive inhibition, as found with rat liver mitochondria[22].

Finally, in oxygraphic tests it was found that the inhibition by palmityl-CoA of the ADP-stimulated respiration of *C. utilis* mitochondria was relieved by increasing concentrations of ADP or uncouplers, a result pointing to the absence of side-effects on the coupling mechanism of oxidative phosphorylation and to the competitive inhibition by palmityl-CoA of the ADP translocation. Similar results have been recently reported for mammalian mitochondria[35–37]. However, it must be recalled that,

TABLE VI

EFFECT OF INHIBITORS ON ADP TRANSLOCATION

[^{14}C]ADP-loaded *C. utilis* mitochondria were preincubated in the standard medium (2 mg protein/ml medium) with atractyloside, bongkrekic acid, carboxyatractyloside and palmityl-CoA at 20 °C for 30 s, then cooled at 0 °C. Exchange was initiated by 400 μM ADP and stopped after 30 s by millipore filtration.

Expt No.	*Inhibitor (nmoles · mg protein^{-1})*	*Inhibition of exchange (%)*
1	Carboxyatractyloside (2.2)	50
	Bongkrekic acid (2.8)	50
2	Atractyloside (23)	53
	Bongkrekic acid (1.8)	28
	Atractyloside (23) + bongkrekic acid (1.8)	51
3	Atractyloside (1)	13
	Carboxyatractyloside (1)	15
	Atractyloside (1) + carboxyatractyloside (1)	30
	Palmityl-CoA (1.5)	10
	Carboxyatractyloside (1) + palmityl-CoA (1.5)	21

in contrast to atractyloside, carboxyatractyloside and bongkrekic acid, palmityl-CoA inhibits other processes than the ADP transport in mitochondria, for instance the malate and citrate transport[39] and the pyridine nucleotide transhydrogenation[40].

The additivity of inhibitor effects has been examined at low concentrations of inhibitors. As shown in Table VI, the effects of atractyloside and carboxyatractyloside were additive, but not those of atractyloside and bongkrekic acid. The inhibitions due to palmityl-CoA and carboxyatractyloside were only partially additive. These results suggest that atractyloside and carboxyatractyloside exert their inhibitory effect by binding to the same site.

(5) Binding properties of [^{35}S]carboxyatractyloside, [^{35}S]atractyloside, and [^{14}C]bongkrekic acid

[^{35}S[Carboxyatractyloside was used to investigate correlations between carboxyatractyloside inhibition and carboxyatractyloside binding and to assess the degree of competition between [^{35}S]carboxyatractyloside and other specific inhibitors for binding to mitochondria of *C. utilis*. As shown in Fig. 7, both the inhibition curve and the binding curve are characterized by a sigmoidal shape, suggesting co-operative interactions for carboxyatractyloside binding. The two curves are virtually superimposable. Similar observations have been reported for binding of [^{35}S]carboxyatractyloside to rat liver mitochondria[22]. The amount of carboxyatractyloside bound at saturation was about 0.5–0.6 nmole per mg of protein and the apparent K_d value 12 to 15 nM.

The kinetics of carboxyatractyloside binding to *C. utilis* mitochondria have been studied at 0 °C (Fig. 8). They are characterized by a rapid phase, corresponding

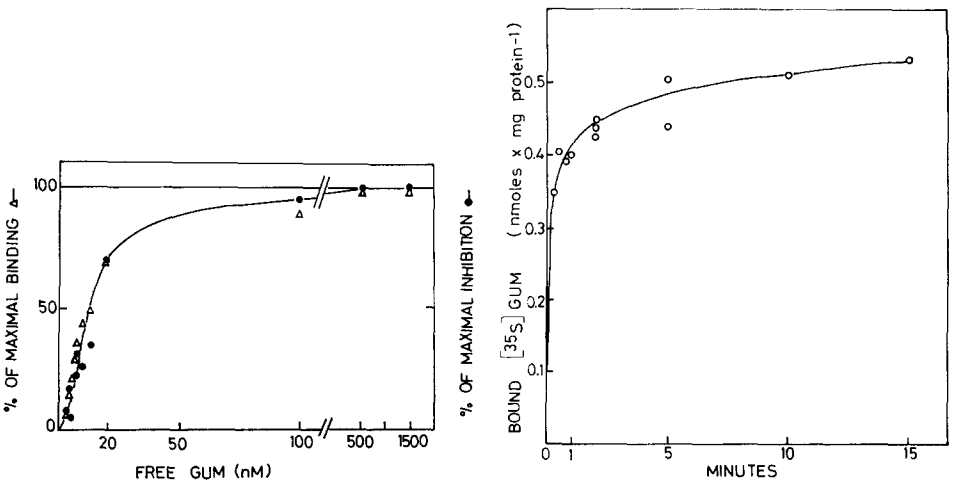

Fig. 7. Correlation between the binding of carboxyatractyloside (gummiferin, (GUM)) to *C. utilis* mitochondria and its inhibitory effect on ADP translocation. (1) ADP translocation: [^{14}C]ADP-loaded mitochondria (4 mg protein) were preincubated for 45 min at 0 °C in the standard medium with increasing concentrations of carboxyatractyloside. Exchange was initiated by addition of 230 μM ADP and stopped after 20 s by millipore filtration. (2) Carboxyatractyloside binding: *C. utilis* mitochondria treated by unlabelled ADP in the same way as the [^{14}C]ADP-loaded mitochondria were incubated for 45 min at 0 °C in the standard medium with increasing concentration of [^{35}S]carboxyatractyloside. Incubation was stopped by centrifugation. The mitochondrial pellets were dissolved in 1 ml of formamide at 180 °C and their radioactivity was determined by liquid scintillation.

Fig. 8. Kinetics of [^{35}S]carboxyatractyloside ([^{35}S]GUM) binding to *C. utilis* mitochondria. Mitochondria (1 mg protein) were incubated at 0 °C with 2 μM [^{35}S]carboxyatractyloside in 1.2 ml of standard medium. Binding was stopped by millipore filtration. Filters were washed twice with 5 ml of cold medium and their radioactivity was counted.

to 70% of the maximal binding in less than 15 s, which is followed by a much slower phase, the maximal inhibition being reached only after 5 min.

Preliminary experiments aimed to test the nature of the carboxyatractyloside receptor have revealed a sensitivity to heating, suggesting that proteins are an integral part of its structure (Fig. 9). A plot of the inhibition rate constant k against $1/T$ allowed us to calculate an activation energy of 88 kcal/mole for the heat denaturation of the carboxyatractyloside binding sites.

In agreement with data obtained with mammalian mitochondria[22], [^{35}S]-carboxyatractyloside binding to *C. utilis* mitochondria was inhibited competitively by atractyloside and non-competitively by bongkrekic acid; it was only slightly affected by high concentrations of ADP (100 μM) (Fig. 10A).

The affinity of [^{35}S]atractyloside for *C. utilis* mitochondria was much lower than that of [^{35}S]carboxyatractyloside and markedly decreased by low concentrations of carboxyatractyloside (Fig. 10B).

Binding assays carried out with [^{14}C]bongkrekic acid indicated a pseudo-saturation at about 0.4 nmole/mg protein for a first set of strong binding sites and a K_d of 15 nM, values which are similar to those found for carboxyatractyloside. Carboxyatractyloside inhibited bongkrekic acid binding (Fig. 10C).

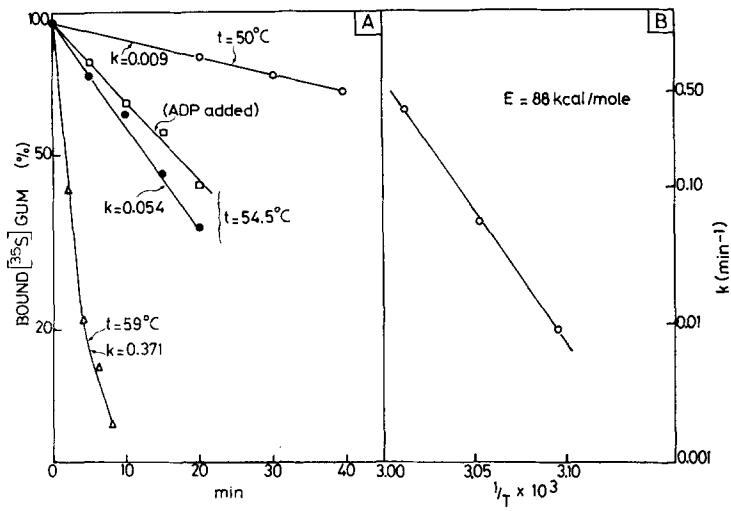

Fig. 9. Effect of temperature on the [^{35}S]carboxyatractyloside ([^{35}S]GUM) binding capacity of *C. utilis* mitochondria. (A) Mitochondria (1 mg protein) were incubated at the indicated temperatures for different periods of time in 1 ml of 0.15 M KCl, 10 mM MOPS and 1 mM EDTA. After cooling, 4 μM [^{35}S]carboxyatractyloside was added to the suspensions and incubated for 45 min at 0 °C. The incubation was ended by centrifugation and the radioactivity of the pellet counted. (B) The rate constants for the heat inactivation of carboxyatractyloside sites (calculated from A) are plotted against $1/T$.

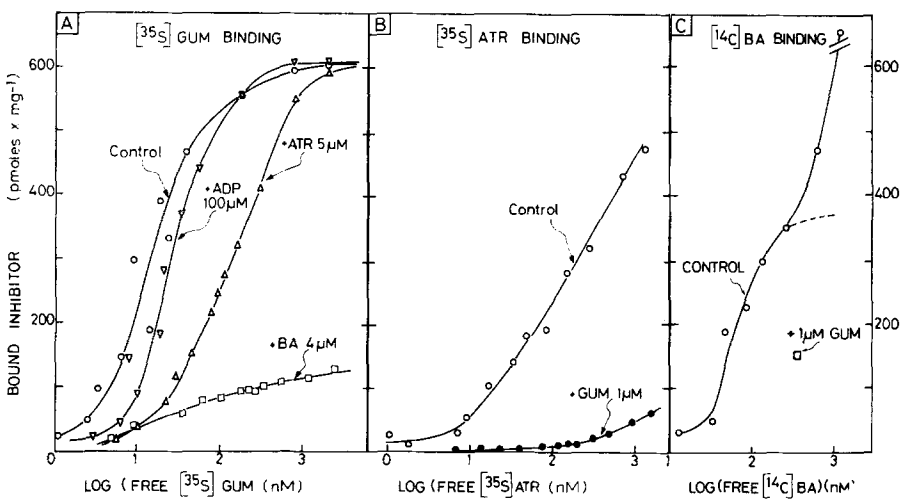

Fig. 10. (A) [^{35}S]Carboxyatractyloside ([^{35}S]GUM) binding to *C. utilis* mitochondria. Effect of ADP, atractyloside and bongkrekic acid. Mitochondria (3.9 mg protein) were preincubated for 2 min at 20 °C in series of tubes containing 4.6 ml of standard medium in the absence (control) or the presence of ADP, atractyloside (ATR) or bongkrekic acid (BA). After cooling, increasing concentrations [^{35}S]carboxyatractyloside were added to each series of tubes and incubation was carried out for 45 min at 0 °C. After centrifugation, the mitochondrial pellets were assayed for radioactivity. (B) [^{35}S]Atractyloside ([^{35}S]ATR) binding. Effect of carboxyatractyloside. Same experimental procedure as in A. In this case, preincubation was carried out without (control) or with carboxyatractyloside (GUM). (C) [^{14}C]Bongkrekic acid ([^{14}C]BA) binding. Incubation with [^{14}C]bongkrekic acid was carried out at 25 °C for 5 min.

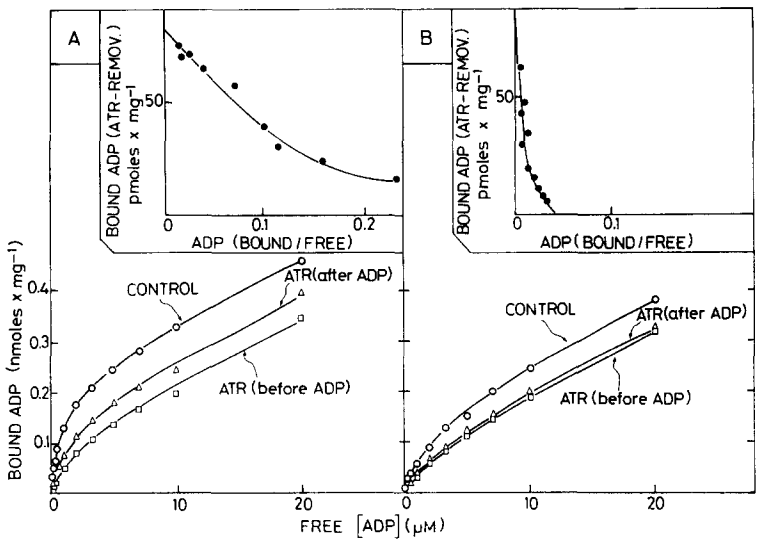

Fig. 11. [^{14}C]ADP binding to *C. utilis* mitochondria depleted of adenine nucleotides. (A) Mitochondria (1.2 mg protein/ml) were depleted of their endogenous adenine nucleotides by incubation in a standard incubation medium supplemented with 10 mM phosphate, pH 6.8, and 10 mM $MgCl_2$, for 15 min at 30 °C, sedimented by centrifugation and resuspended in a standard medium. The binding assay was carried out in series of tubes containing 2 ml of standard medium and increasing concentrations of [^{14}C]ADP. Atractyloside (ATR) (200 μM) was either absent (control) or added before or after ([^{14}C]ADP. The incubation was of 2 min at 0 °C. After centrifugation, the radioactivity of the mitochondrial pellet was assayed. (B) The procedure was the same as in A except that mitochondria were treated twice by phosphate and Mg^{2+} and were kept frozen for one night. In both cases, the fraction of bound ADP which may be removed by addition of atractyloside has been plotted against ADP (bound/free) (see inserts).

(6) *Binding of* [^{14}C]*ADP to C. utilis mitochondrial membranes*

A convenient way to measure the amount of [^{14}C]ADP bound to the mitochondrial specific translocator is to estimate the amount of bound [^{14}C]ADP removed by addition of atractyloside[41,42]. To lower the fraction of [^{14}]CADP incorporated in the mitochondrial matrix with respect to the [^{14}C]ADP bound to the membrane, one may deplete the mitochondria from their endogenous adenine nucleotides[41,42]. As mentioned above in this paper, a simultaneous addition of phosphate and Mg^{2+} to *C. utilis* mitochondria at room temperature leads to an extensive leakage of internal adenine nucleotides. The same depletion procedure has been followed to study the [^{14}C]ADP binding. Results of two experiments are presented in Fig. 11. In Fig. 11A, *C. utilis* mitochondria were incubated for 15 min at 30 °C in a standard incubation medium supplemented with 10 mM phosphate and 10 mM $MgCl_2$, centrifuged and assayed for ADP binding. In Fig. 11B, the phosphate–Mg^{2+} treatment was repeated twice, followed by freezing of mitochondria at −20 °C for one night. As shown by the percentage of inhibition due to atractyloside added either before [^{14}C]ADP (inhibition of exchange) or after [^{14}C]ADP (inhibition of binding), a repeated phosphate–Mg^{2+} treatment followed by freezing resulted in mitochondria in which the atractyloside-sensitive uptake of [^{14}C]ADP was essentially related to a specific ADP carrier binding (Fig. 11B). From the Scatchard plot (inserts Figs 11A and 11B) of

bound atractyloside-removable [^{14}C]ADP, a maximal amount of specific ADP binding sites of about 0.08 nmole per mg protein may be deduced. This value is of the same order as that reported recently by Kolarov *et al.*[43] in the case of *S. cerevisiae*. In other experiments, values exceeding 0.15 nmole per mg protein were found. No attempt was made to decompose the Scatchard curves (Fig. 11) into slopes of low and high affinity. However, it is clear from the apparent K_d related to the main portion of the curves that the affinity for ADP was markedly lower after two phosphate–Mg^{2+} consecutive treatments than after only one treatment.

(7) Effect of growth conditions on ADP translocation and carboxyatractyloside (gummiferin) binding

Repressibility of mitochondrial development by glucose in yeast is a well-documented phenomenon (for review, see ref. 44). The mitochondrial protein synthesizing machinery can also be inhibited by specific inhibitors like chloramphenicol[45–47].

TABLE VII

INFLUENCE OF GROWTH CONDITIONS ON THE ADP TRANSPORT AND THE BINDING OF [^{35}S]CARBOXYATRACTYLOSIDE

C. utilis cells were grown as indicated in Methods in a medium supplemented with either 1.5% glucose, 10% glucose, 3% ethanol with or without chloramphenicol (4 mg/ml). ADP translocation and [^{35}S]carboxyatractyloside binding were assayed with standard methods at 0 °C (see legend of Fig. 7). The [^{14}C]ADP binding, atractyloside-removable, was determined as in Fig. 11. The [^{35}S]carboxyatractyloside concentration in the binding assay was 2 μM. The rate of exchange was calculated for the exchange of the added ADP with internal ADP *plus* ATP.

Growth conditions	*Cytochrome aa_3** ($nmoles \cdot g^{-1}$)	*Rate of exchange* ($\mu moles \cdot min^{-1} \cdot g^{-1}$)	[^{35}S]*Carboxyatractyloside binding* ($nmoles \cdot mg^{-1}$)	[^{14}C]*ADP binding, atractyloside removable* ($nmoles \cdot mg^{-1}$)
1.5% glucose	14.7	3.5	0.85	0.15
10% glucose	3.1	1.0	0.82	—
3% ethanol	35.3	11.5	0.64	0.16
3% ethanol + chloramphenicol	8.1	2.4	0.76	—

* Cytochrome aa_3 is given in nmoles per g of cells (dry weight).

The ability of *C. utilis* mitochondria to transport [^{14}C]ADP and to bind [^{35}S]carboxyatractyloside has been examined with mitochondria prepared from cells grown in the presence of (1) 1.5% glucose, (2) 10% glucose, (3) 3% ethanol, (4) 3% ethanol *plus* chloramphenicol (Table VII). In all cases, the size of the pool of endogenous adenine nucleotides was of the same order of magnitude. However, the velocity of ADP transport, as well as the rate constant (not shown in Table VII), was maximal for mitochondria prepared from yeast grown in the presence of ethanol. These values were lowered from 3 to 10 times, while remaining sensitive to atractyloside, when the yeast was grown in the presence of glucose or of chloramphenicol, *i.e.* under conditions which repress the development of aerobic metabolism. In all cases, there

was a strict parallelism between the decrease in the ability to transport ADP and the decrease in cytochrome aa_3, pointing to the fact that the functioning of the ADP carrier is dependent at least partially on membrane components synthesized by the mitochondrial machinery. By contrast, the number of carboxyatractyloside binding sites and of the atractyloside-removable ADP binding sites was not modified.

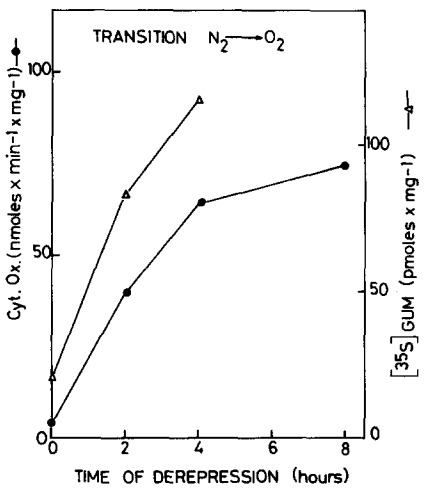

Fig. 12. Effect of derepression on the cytochrome oxidase activity and the [^{35}S]carboxyatractyloside ([^{35}S]GUM) binding capacity of *S. cerevisiae* mitochondria. *S. cerevisiae* (IL 8–8C) cells were grown at 28 °C under anaerobiosis in a medium made of peptone and yeast extract supplemented with ergosterol (20 mg/l), Tween 80 (5 ml/l) and 10% glucose as detailed in Methods, and harvested. After three successive washings, they were derepressed by incubation under aerobiosis in 50 mM potassium phthalate, 50 mM sodium succinate, pH 5, and 0.1% glucose. The cell concentration was 4 mg (dry weight)/ml. Air was flushed in the medium at a rate of 1 l/min per l of medium. Cells were harvested at different times and their mitochondria extracted. Carboxy[^{35}S]atractyloside binding was assayed by incubating mitochondria (4.5 mg protein) in 5 m of standard medium with 2 μM [^{35}S]carboxyatractyloside for 45 min at 0 °C.

Complementary studies bearing on the effect of strict anaerobiosis on [^{35}S]-carboxyatractyloside and [^{14}C]ADP binding were carried out with *S. cerevisiae*, a facultative anaerobe which can grow in the absence of O_2 and with glucose as a source of carbon in a medium supplemented with ergosterol and Tween 80. In this case, the carboxyatractyloside binding capacity and the cytochrome oxidase activity were virtually abolished by anaerobiosis; they were partially restored upon aeration of the yeast suspension for a few hours at 28 °C (Fig. 12). The number of atractyloside-sensitive ADP binding sites followed the same variation (not shown on Fig. 12); in anaerobiosis it was less than 0.005 nmole per mg protein; in aerobiosis it amounted to 0.11 nmole per mg protein. Corroborating the above data on anaerobiosis, it was found that mitochondria from *C. utilis* grown in semi-anaerobiosis (growth in fermentor jars with stirring, but without air bubbling) lose part of their carboxyatractyloside binding sites (about 50%).

DISCUSSION

Results presented in this paper on the general properties of the mitochondrial ADP transport in *C. utilis* allow us to confirm previous reports by Onishi *et al.*[23] on *S. carlsbergensis* and by Kolarov *et al.*[43] on *S. cerevisiae*, showing a broad similarity of properties of adenine nucleotide transport in mammalian and yeast mitochondria. The ADP transport in yeast and mammalian mitochondria is an exchange–diffusion process characterized by a complete exchangeability of the pool of mitochondrial adenine nucleotides, a marked specificity for ADP, a high sensitivity to specific inhibitors, namely atractyloside, gummiferin (carboxyatractyloside), bongkrekic acid and palmityl-CoA. Like in rat liver mitochondria, added ADP is exchanged in preference with internal ATP. This peculiarity, under conditions of oxidative phosphorylation, results in an increase of the ATP/ADP ratio in the extramitochondrial space with respect to the matrix space.

However, as revealed by the present study, the mitochondrial adenine nucleotide translocation in *C. utilis* differs in a few features from that in rat liver. (1) The specificity of translocation in *C. utilis* mitochondria is not restricted to ADP and ATP or to adenine analogues, but it extends also to UDP. Actually, an analogous situation is encountered with brown adipose tissue mitochondria, which appear to be permeable to GDP[48], a nucleotide which plays in these mitochondria a role of a prime importance in the substrate level phosphorylation and in the GTP–AMP transphosphorylation[49]. Similarly, the entry of UDP in *C. utilis* mitochondria might be linked to some metabolic regulation. Besides, it is interesting to note that the concentration of UDP *plus* UTP is markedly higher in yeast than in rat liver mitochondria[23]. (2) Inhibitors of ADP translocation are 2 to 4 times less active in *C. utilis* than in rat liver mitochondria. (3) There is no transition point in the Arrhenius plot of the rate of translocation by yeast mitochondria in contrast to liver mitochondria. While these differences may result from alterations of the inner mitochondrial membrane due to the method of preparation of yeast mitochondria, they also may reflect genuine properties of the ADP translocation system depending on the source of mitochondria.

There have been in recent years an increasing number of papers on the genetic approach to adenine nucleotide translocation in yeast mitochondria. Mutants deficient in the transport of ADP have been isolated and their defect in terms of loss of affinity for ADP has been characterized[43,50–52]. In contrast, little has been reported on the effect of physiological manipulation on the activity and the properties of the mitochondrial ADP translocator. In a recent paper, Perkins *et al.*[52] have shown that the uptake of ATP by fully functional mitochondria of *S. cerevisiae* and by anaerobic mitochondrial structures of the same strain was similar, although the K_m for the atractyloside-sensitive uptake was higher. These data were interpreted as a lower affinity of the ADP carrier for atractyloside in anaerobic mitochondria. Groot *et al.*[53] have reported that promitochondria of another strain of *S. cerevisiae* were able to catalyze an atractyloside-sensitive ATP–P_i exchange, suggesting that promitochondria are still able to transport ATP; nevertheless, as judged by the very high concentration of atractyloside (500 μM) required to inhibit the exchange in their experiment, the sensitivity of ATP transport to atractyloside in promitochondria might be very low. Data presented in this paper show that in mitochondrial particles isolated from *S. cerevisiae* grown under strict anaerobiosis or from *C. utilis* grown under semi-an-

aerobiosis, the number of carboxyatractyloside binding sites and ADP binding sites is markedly decreased. The capacity to bind carboxyatractyloside and ADP is restored when cells are aerated. A minimal hypothesis to explain these data is that anaerobiosis blocks the synthesis of membrane components necessary for the integration and the functioning of the carboxyatractyloside receptor and of the ADP translocator. Comparison of our results with those of Perkins *et al.*[52] and Groot *et al.*[53] appears not easy, because of the usual contamination of anaerobic promitochondria by other subcellular membranes. Much more conclusive are experiments carried out with *C. utilis* grown in aerobiosis under normal (ethanol) or repressed (glucose) conditions.

A very significant difference in the rate of ADP transport was found in mitochondria of *C. utilis* according to the aerobic growth conditions. For instance, mitochondria isolated from *C. utilis* grown on 3% ethanol were able to transport ADP 3 to 10 times faster than mitochondria from yeast grown on glucose. This difference in the rate of ADP transport is apparently not related to the affinity of mitochondria for ADP, nor to the size of the mitochondrial pool of adenine nucleotides, nor to the number of atractyloside-removable ADP binding sites, all of which are similar under the above-mentioned growth conditions. From data of Table VII, it may be calculated that the turnover number of the ADP transport per ADP site is of the order of 72 min^{-1} at 0 °C when yeast has been grown on 3% ethanol and 23 min^{-1} when ethanol is replaced by 1.5% glucose. Even larger variations might be obtained when 1.5% glucose is replaced by 10% glucose. Assuming that the turnover of a carrier is related to its mobility in the membrane, the above-mentioned changes in the turnover number of the ADP translocator according to growth conditions may be due to a variable degree of fluidity of the inner mitochondrial membrane. As shown in a previous paper[54], increasing the glucose concentration in the growth medium of *C. utilis* results in a decreased value of the phospholipid to protein ratio in mitochondria. Although further work is needed to specify the modifications of the phospholipid–protein architecture of the mitochondrial membrane upon glucose repression, it is tempting to speculate that these modifications are responsible for the decrease in the mobility of the ADP translocator.

It is noteworthy that in all conditions tested, the carboxyatractyloside binding capacity varies in parallel with the specific ADP binding capacity. This finding strengthens a previous statement[42] that carboxyatractyloside and ADP bind to the same receptor or at least in close proximity.

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