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# Biogenesis of Mitochondria*

## A REQUIREMENT FOR MITOCHONDRIAL PROTEIN SYNTHESIS FOR THE FORMATION OF A NORMAL ADENINE NUCLEOTIDE TRANSPORTER IN YEAST MITOCHONDRIA

By J. M. HASLAM,† MARGARET PERKINS and ANTHONY W. LINNANE
*Department of Biochemistry, Monash University, Clayton, Vic.* 3168, *Australia*

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1. Parameters of ATP uptake by fully functional *Saccharomyces cerevisiae* mitochondria, including kinetic constants, binding constants and sensitivity to atractylate, closely resemble those of mammalian mitochondria. Scatchard plots of atractylate-sensitive adenine nucleotide binding indicate two distinct sites of high affinity (binding constant, $K_D' = 1\,\mu M$), and low affinity (binding constant, $K_D'' = 20\,\mu M$) in the ratio 1:3. Uptake has high Arrhenius activation energies (+35 and +57 kJ/mol), above and below a transition temperature of 11°C. Atractylate-insensitive ATP uptake is apparently not saturable and has a low Arrhenius activation energy (6 kJ/mol), suggesting a non-specific binding process. 2. Kinetic and binding constants for ATP uptake are not significantly changed in catabolite-repressed or anaerobic mitochondrial structures. 3. Inhibition of the mitochondrial protein-synthesizing system by growth of cells in the presence of erythromycin, or loss of mitochondrial DNA by mutation profoundly alters the adenine nucleotide transporter. ATP uptake becomes completely insensitive to atractylate, and the high-affinity binding site is lost. However, the adenine nucleotide transporter does not appear to be totally eliminated, as a moderate amount of saturable low-affinity ATP binding remains. 4. It is concluded that products of the mitochondrial protein-synthesizing system, probably coded by mitochondrial DNA, are required for the normal function of the adenine nucleotide transporter.

The properties of the adenine nucleotide transporter of mammalian mitochondria have been extensively investigated, and were reviewed by Klingenberg (1970). The carrier is highly specific for ADP and ATP (Pfaff & Klingenberg, 1968), catalyses a 1:1 exchange between exogenous and endogenous ADP (Pfaff *et al.*, 1965), and is inhibited by atractylate (Bruni *et al.*, 1964). Weidemann *et al.* (1970) have shown that the uptake of ADP and ATP by exchange is minimal in mitochondria that have first been depleted of endogenous nucleotides by incubation with arsenate. By using this system Weidemann *et al.* (1970) have identified two atractylate-sensitive ATP-binding sites of unequal affinity in mammalian mitochondria. However, attempts to purify an atractylate-sensitive adenine nucleotide-binding protein from mammalian mitochondria have so far been unsuccessful (Klingenberg, 1970; Chavin, 1971). An alternative approach is to use the yeast *Saccharomyces cerevisiae*, whose advantages in the study of mitochondrial membrane functions are well recognized (for reviews see Linnane & Haslam, 1970; Linnane *et al.*, 1972). In particular, the composition of mitochondria in *S. cerevisiae* is subject to physiological control by catabolite repression and anaerobiosis and to genetic control by the selection of nuclear and mitochondrial mutants.

Previous work has led to the partial characterization of the adenine nucleotide transporter of yeast mitochondria. Ohnishi *et al.* (1967) reported that the exchange of adenine nucleotides by mitochondria from *Saccharomyces carlsbergensis* is inhibited by atractylate, and has similar properties to that of mammalian mitochondria, except that the yeast system is more active at low temperatures. Kolarov *et al.* (1972*a*) report that the mitochondrial ATP transporter is apparently normal in a cytoplasmic respiratory-deficient mutant of *S. cerevisiae*, but is markedly changed in the nuclear $op_1$ mutant (Kolarov *et al.*, 1972*b*). In the present work the uptake of ATP by *S. cerevisiae* mitochondria is further characterized by using the assay system of Weidemann *et al.* (1970) and by investigating the effects of varying the mitochondrial membrane composition by physiological and genetic means. The results show that the formation and properties of the transporter are subject to

* This paper is no. 32 in the series. No. 31 is Perkins *et al.* (1973).

† Present address: Department of Biochemistry, University of Liverpool, Liverpool, L69 3BX, U.K.

manipulation, and may lead to a further understanding of the nature and mechanism of the carrier system. A preliminary report of some of this work has been published (Perkins *et al.*, 1972).

## Experimental

### *Preparation of mitochondria*

The growth of yeast and the isolation of the five distinctive types of yeast mitochondria are described in the preceding paper (Perkins *et al.*, 1973). In certain experiments the mitochondria were further purified by centrifuging on a discontinuous sorbitol gradient as described by Watson *et al.* (1970).

### *Criteria of mitochondrial purity and intactness of mitochondria*

*Electron microscopy.* Electron microscopy of mitochondria was performed as described by Watson *et al.* (1970), except that the concentration of glutaraldehyde used in fixation was increased to 2%.

*Cytochrome spectra.* Reduced minus oxidized difference spectra of isolated mitochondria were determined in a Cary model 14 spectrophotometer as described by Chance (1957).

*Sucrose-impermeable space.* The sucrose-impermeable space was determined as described previously (Perkins *et al.*, 1973), except that mitochondria were incubated in ATP-uptake medium.

### *Assay of ATP uptake*

The measurement of the binding of ATP to atractylate-sensitive mitochondrial sites is complicated by the considerable contribution to the total uptake from ATP exchange with endogenous nucleotides (Weidemann *et al.*, 1970). This makes the comparison of the properties of different types of yeast mitochondria particularly difficult, as the mitochondria have different pools of endogenous adenine nucleotides. Accordingly, to minimize the contribution of nucleotide exchange, mitochondria were depleted of endogenous nucleotides by preincubation in a medium containing arsenate (Weidemann *et al.*, 1970). Mitochondria were suspended at a concentration of about 5mg of protein/ml and preincubated for 45min at 4°C in a medium containing sorbitol (0.5M), bovine serum albumin (2mg/ml), 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonate (10mM), sodium arsenate (5mM), EDTA (1mM) and $MgCl_2$ (5mM), final pH6.5. The mitochondria were separated by centrifugation and then washed twice by centrifugation in the same medium lacking arsenate and $MgCl_2$. The concentrations of AMP, ADP and ATP in depleted and non-depleted mitochondria were determined by the methods of Adam (1965) and Lamprecht & Trautschold (1965).

Mitochondria (0.5–1.0mg of protein) were then incubated at 30°C in 3ml of medium containing 2-*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonate (10mM), bovine serum albumin (2mg/ml), sorbitol (0.5M), ethanedeoxybis(ethylamine)tetra-acetate (EGTA; 1mM) and oligomycin (30$\mu$g/ml), final pH6.5. Atractylate was added 2min before the addition of [$^{14}$C]ATP (0.025$\mu$Ci). Uptake was terminated by filtration through a Millipore filter (0.45$\mu$m pore size) under reduced pressure in less than 2s. The mitochondria were washed on the filter with 6ml of ice-cold incubation medium. The filters were dried and radioactivity counted in 5ml of toluene scintillator fluid as described previously (Perkins *et al.*, 1973). Incubations of 5, 10, 20, 30 and 60s were performed at 30°C. Initial rates of uptake cannot be determined as uptake exceeds 50% of the final equilibrium extent even at 5s, and equilibrium is reached at 20s with all types of arsenate-depleted mitochondria. Empirical $v_{80}$ rates of uptake were determined as described in the preceding paper (Perkins *et al.*, 1973). Empirical $K_m$ and $V_{80,max.}$ values for the five types of mitochondria are calculated from double-reciprocal plots of $v_{80}$ values at seven different concentrations of ATP in the range 1.5–100$\mu$M for atractylate-sensitive uptake, and at five different concentrations in the range 0.15–100mM for atractylate-insensitive uptake.

Klingenberg and co-workers have chosen the cytochrome *a* content of mitochondria as a convenient index of the amount of mitochondrial inner membrane (Weidemann *et al.*, 1970). In our studies this is not possible, as Types 3, 4 and 5 mitochondria contain no detectable cytochrome *a*, and the amount of cytochrome *a* in Type 2 mitochondria is greatly decreased in comparison with fully functional Type 1 mitochondria. As there is no other convenient marker present in all types of mitochondria our results are expressed per mg of total mitochondrial protein, assayed by the method of Gornall *et al.* (1949).

### *Determination of binding constants*

Binding constants for high-affinity ($K_D'$) and low-affinity ($K_D''$) ATP-binding sites, and the percentage contributions of the two types of binding sites were determined by extrapolations of Scatchard plots of ATP uptake as described by Weidemann *et al.* (1970).

### *Reagents and enzymes*

Atractylate and 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonate were from Calbiochem, San Diego, Calif., U.S.A. [$^{14}$C]ATP, [$^{14}$C]sucrose and $^3H_2O$ were from The Radiochemical Centre, Amersham, Bucks., U.K. Oligomycin, ATP, glucose 6-phosphate dehydrogenase (EC 1.1.1.49), lactate dehydrogenase (EC 1.1.1.27) and pyruvate kinase (EC 2.7.1.40) were from Sigma Chemical Co., St. Louis, Mo., U.S.A.

## Results

### *Adenine nucleotides of mitochondria*

The concentrations of adenine nucleotides in freshly isolated and arsenate-depleted mitochondria of Types 1, 2, 4 and 5 are compared in Table 1. (ADP+ATP) comprise 81% of the adenine nucleotides of freshly isolated, fully functional (Type 1) mitochondria, and are comparable in amount with those of mammalian mitochondria (Weidemann *et al.*, 1970). Preincubation with arsenate results in the conversion of approximately one-half the (ATP+ADP) into AMP, and there is a moderate decrease in total nucleotides, thus diminishing the exchange of exogenous ATP for endogenous nucleotides (Weidemann *et al.*, 1970). Mitochondrial Types 2, 4 and 5 all lack oxidative phosphorylation, and this is reflected in their low contents of (ATP+ADP) and relatively high contents of AMP; the ATP present in these organelles is presumably transported in from the cytoplasm. The total adenine nucleotide contents of Types 2, 4 and 5 mitochondria are much lower than those of fully functional Type 1 mitochondria, and are further lowered by preincubation with arsenate. Arsenate treatment also increases the proportion of AMP in Types 2, 4 and 5.

### *ATP uptake by Type 1 mitochondria*

The time-course for the uptake of low concentrations of ATP (1.5$\mu$M) by arsenate-depleted fully functional Type 1 mitochondria is shown in Fig. 1; uptake is very rapid and reaches equilibrium after about 20s with the mitochondria binding 16pmol of ATP·mg of protein$^{-1}$. In the presence of high concentrations of atractylate (100$\mu$M) the uptake of ATP (1.5$\mu$M) is inhibited by 67%. Table 2 shows that the extent of uptake increases greatly at higher concentrations of ATP, but the sensitivity to atractylate

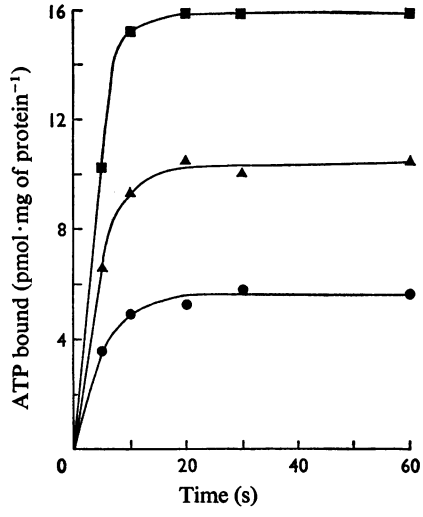



Fig. 1. *Time-course of ATP uptake by arsenate-depleted Type 1 mitochondria*

The uptake of ATP (1.5$\mu$M) by arsenate-depleted Type 1 mitochondria was measured as described in the Experimental section. ■, Total uptake in the absence of atractylate; ●, uptake insensitive to atractylate (0.1mM); ▲, atractylate-sensitive uptake, obtained by subtraction.

Table 1. *Endogenous adenine nucleotide contents of freshly prepared and arsenate-depleted mitochondria*

The preparation of mitochondria and assay of adenine nucleotides (nmol·mg of protein$^{-1}$) is described in the Experimental section. The results represent individual experiments. Treatment (a) represents fresh mitochondria; treatment (b) arsenate-depleted mitochondria.

| | | Adenine nucleotide content (nmol·mg of protein$^{-1}$) | | | |
|---|---|---|---|---|---|
| Mitochondria | Treatment | AMP | ADP | ATP | Total |
| Type 1 | (a) | 2.1 | 3.0 | 6.0 | 11.1 |
| | (b) | 3.3 | 2.0 | 1.8 | 7.1 |
| | (b) | 3.0 | 2.2 | 1.8 | 7.0 |
| Type 2 | (a) | 0.7 | 1.0 | 0.7 | 2.4 |
| | (a) | 0.4 | 0.7 | 0.7 | 1.8 |
| | (b) | 1.1 | 0.4 | 0.1 | 1.6 |
| | (b) | 1.1 | 0.2 | 0.2 | 1.5 |
| Type 4 | (a) | 2.1 | 1.5 | 1.9 | 5.5 |
| | (b) | 2.1 | 0.5 | 1.3 | 3.9 |
| | (b) | 1.5 | 1.0 | 1.3 | 3.8 |
| Type 5 | (a) | 1.5 | 1.5 | 0.5 | 3.5 |
| | (b) | 2.0 | 0.4 | 0.3 | 2.7 |

Table 2. *Inhibition by atractylate of ATP uptake by Type 1 mitochondria*

[$^{14}$C]ATP uptake (pmol·mg of protein$^{-1}$) was measured as described in the Experimental section in the presence of the concentrations of ATP and atractylate indicated.

| ATP ($\mu$M) | Atractylate ($\mu$M) | Uptake of ATP (pmol·mg of protein$^{-1}$) | Inhibition (% of control) |
|---|---|---|---|
| 1.5 | 0 | 16 | 0 |
| 1.5 | 100 | 5.3 | 67 |
| 15 | 0 | 44 | 0 |
| 15 | 100 | 21 | 52 |
| 25 | 0 | 70 | 0 |
| 25 | 100 | 41 | 42 |
| 100 | 0 | 190 | 0 |
| 100 | 100 | 143 | 25 |
| 100 | 500 | 144 | 24 |
| 100 | 2500 | 139 | 27 |

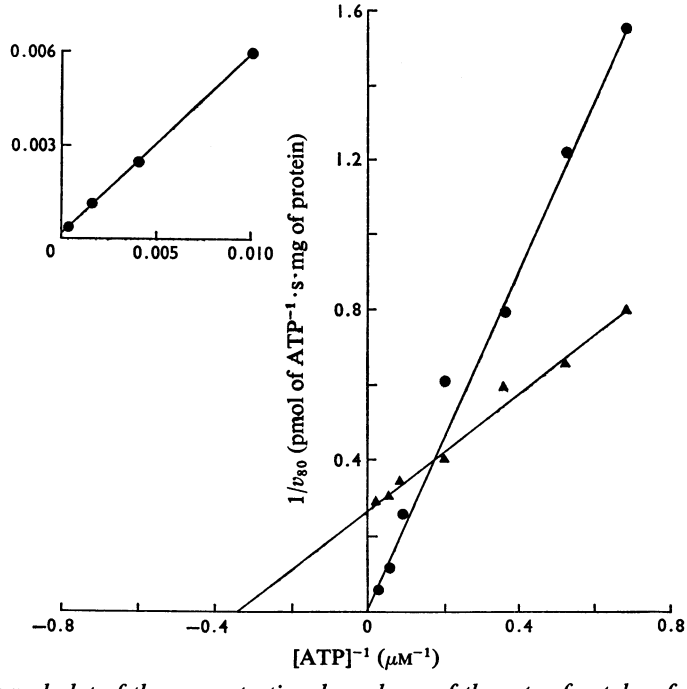



Fig. 2. *Double-reciprocal plot of the concentration-dependence of the rate of uptake of ATP by arsenate-depleted Type 1 mitochondria*

The time-course of uptake of [$^{14}$C]ATP by Type 1 mitochondria (a) in the absence of atractylate, (b) in the presence of atractylate (0.1 mM), and $v_{80}$ rates of uptake were calculated as described in the Experimental section. Double-reciprocal plots are shown of the concentration-dependence of $v_{80}$ rates of uptake, and the inset shows further points for atractylate-insensitive ATP uptake: ●, atractylate-insensitive ATP uptake obtained in (b); ▲, atractylate-sensitive ATP uptake given by the difference between (a) and (b). Calculated kinetic constants for atractylate-sensitive ATP uptake: $K_m = 3\,\mu$M; $V_{80,max.} = 4$ pmol·s$^{-1}$·mg of protein$^{-1}$.

decreases. The inhibition of ATP uptake by atractylate at an ATP concentration of 100 $\mu$M is maximal at 25%, as increasing the concentration of atractylate to 2.5 mM does not significantly increase the inhibition.

Fig. 2 is a Lineweaver–Burk plot of the $v_{80}$ rates of

ATP uptake by arsenate-depleted Type 1 mitochondria for seven different concentrations of ATP in the concentration range 1.5–100 $\mu$M; the inset graph shows four further points for atractylate-insensitive uptake of ATP in the concentration range 100–2000 $\mu$M. The plot for atractylate-sensitive $v_{80}$ rates of ATP uptake approximates to a straight line, giving an empirical $K_m$ value of 3 $\mu$M. Atractylate-insensitive ATP uptake also gives a straight-line plot which almost intersects the origin, and may represent a non-saturable process ($K_m > 2$ mM).

Scatchard plots of the equilibrium extents of ATP binding by Type 1 mitochondria are given in Fig. 3. The plot of atractylate-insensitive ATP binding approximates to a vertical straight line, indicating a non-saturable process. The Scatchard plot of atractylate-sensitive binding gives a compositive curve that can be resolved into two sites of unequal affinity. By using the extrapolations of Weidemann *et al.* (1970), it was calculated that the first site has a $K_D'$ value of 1 $\mu$M and binds approximately 25% of the ATP, and the second has a $K_D''$ value of 20 $\mu$M and binds 75% of the ATP. Total atractylate-sensitive ATP binding is 40 pmol·mg of protein$^{-1}$.

Weidemann *et al.* (1970) showed that, although the uptake of adenine nucleotides by exchange is greatly diminished by arsenate treatment of mitochondria, exchange still comprises a significant proportion of the total uptake. Since our arsenate-depleted yeast mitochondria contain moderate concentrations of (ADP+ATP), it was necessary to determine whether the additional uptake due to exchange could modify the apparent $K_m$ and $K_D$ values for atractylate-sensitive and atractylate-insensitive ATP uptake.

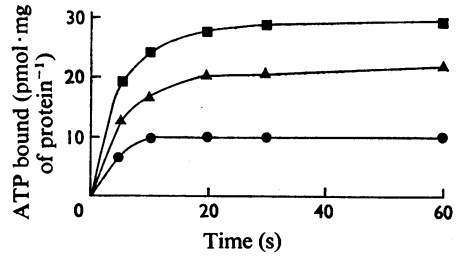



Fig. 4. *Time-course of uptake of ATP by freshly prepared Type 1 mitochondria*

The uptake of ATP (1.5 $\mu$M) by freshly prepared Type 1 mitochondria in the presence and in the absence of atractylate (0.1 mM) was measured as described in the Experimental section: ■, total uptake; ▲, atractylate-sensitive uptake; ●, atractylate-insensitive uptake.

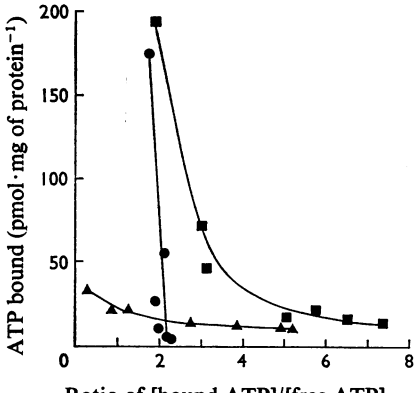



Fig. 3. *Scatchard plots of the uptake of ATP by arsenate-depleted Type 1 mitochondria*

The equilibrium extents of uptake of [$^{14}$C]ATP at a concentration of 1.5–100 $\mu$M were determined in the presence and in the absence of atractylate (0.1 mM) as described in the Experimental section. In all cases equilibrium was reached by 60s: ■, total uptake; ▲, atracylate-sensitive uptake; ●, atractylate-insensitive uptake. Calculated binding constants for atractylate-sensitive binding: $K_D' = 1$ $\mu$M (25% of total binding); $K_D'' = 20$ $\mu$M (75% of total binding); total binding = 40 pmol·mg of protein$^{-1}$.

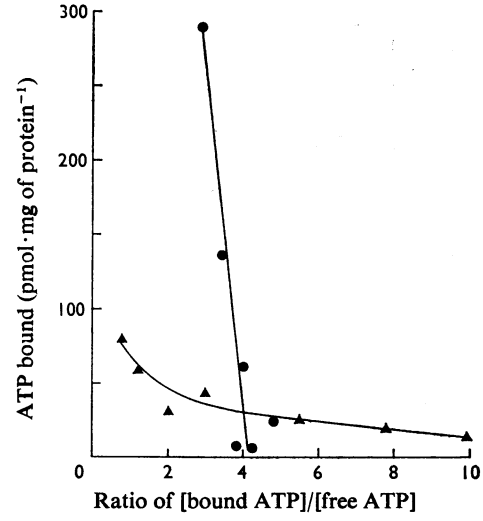



Fig. 5. *Scatchard plot of the uptake of ATP by freshly-prepared Type 1 mitochondria*

Conditions are as described in Fig. 3. ▲, Atractylate-sensitive binding; ●, atractylate-insensitive binding. Calculated binding constants for atractylate-sensitive binding: $K_D' = 2$ $\mu$M (30% of total binding); $K_D'' = 28$ $\mu$M (70% of total binding); total binding = 100 pmol·mg of protein$^{-1}$.

Kinetic and binding constants were determined for the uptake of ATP by freshly prepared mitochondria in which exchange is the predominant mode of uptake. The time-course for the uptake of ATP (1.5 $\mu$M) by freshly prepared mitochondria is shown in Fig. 4. Uptake is very rapid and the atractylate-insensitive portion is complete by 20s as in arsenate-depleted mitochondria. Atractylate-sensitive uptake of ATP is slower than in arsenate-depleted mitochondria, and is not complete until 60s, and the total uptake of 28pmol of ATP.mg of protein$^{-1}$ is nearly twice that found in the arsenate-depleted mitochondria, but the $K_m$ value of 3 $\mu$M is very similar. The slower rate and increased extent of ATP uptake by fresh yeast mitochondria compared with arsenate-depleted organelles are also observed in intact mammalian mitochondria, and reflect the preponderance of uptake by exchange (Pfaff *et al.*, 1969; Weidemann *et al.*, 1970). A Scatchard plot of ATP uptake by freshly prepared yeast mitochondria is shown in Fig. 5. The binding constants, $K_D' = 2\mu$M, $K_D'' = 28\mu$M, for atractylate-sensitive ATP uptake are very similar to these for arsenate-depleted mitochondria, and indicate that variations in the endogenous adenine nucleotide content of the different types of mitochondria do not seriously affect either the kinetic or binding parameters of ATP uptake. Nevertheless, we decided to use arsenate-depleted mitochondria in our comparative studies, as freshly prepared respiratory-deficient Types 2, 4 and 5 mitochondria contain much lower concentrations of adenine nucleotides than do respiratory-competent Types 1 and 3 organelles, whereas arsenate depletion results in all types of organelles having very low concentrations of (ADP+ATP).

Arrhenius plots of the temperature-dependence of the $v_{80}$ rates of the uptake of ATP (2 $\mu$M) by arsenate-depleted Type 1 mitochondria are shown in Fig. 6. The activation energy of atractylate-insensitive uptake of ATP by Type 1 and Type 5 mitochondria is very low (6kJ/mol). The atractylate-sensitive uptake of ATP by Type 1 mitochondria shows a discontinuity at 11°C with activation energies at +35 and +57kJ/mol above and below the transition temperature.

*Uptake of ATP by modified mitochondrial Types 2, 3, 4 and 5*

The kinetics of ATP uptake by the four modified types of yeast mitochondria follows a similar time-course to that of Type 1 mitochondria, enabling the determination of empirical kinetic constants from

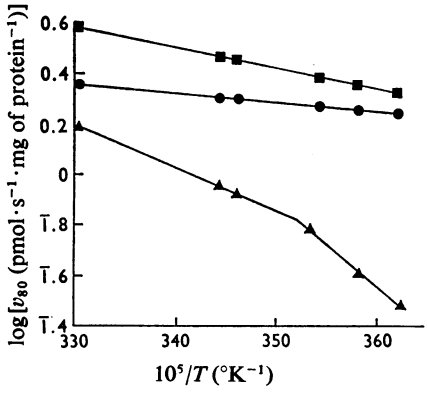



Fig. 6. *Arrhenius plots of the temperature-dependence of the rates of uptake of ATP by arsenate-depleted Type 1 mitochondria*

$v_{80}$ rates of uptake of ATP (2 $\mu$M) by arsenate-depleted Type 1 mitochondria were determined at six temperatures in the range 4–30°C, and are given as an Arrhenius plot. ■, Total ATP uptake; ●, uptake insensitive to atractylate (0.1mM); ▲, atractylate-sensitive ATP uptake. Atractylate-insensitive ATP uptake has an Arrhenius activation energy of +6kJ/mol, and atractylate-sensitive ATP uptake has activation energies of +35 and +57kJ/mol respectively above and below a transition temperature of 11°C.

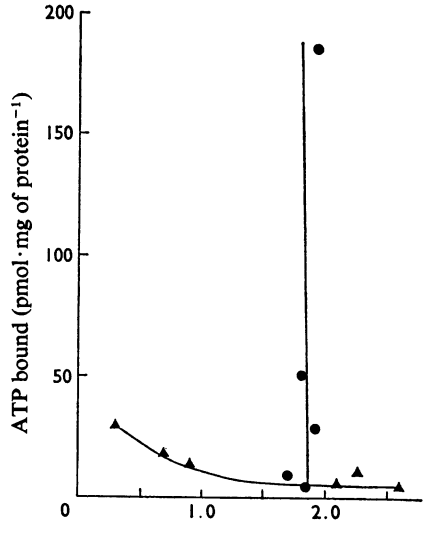



Fig. 7. *Scatchard plot of the uptake of ATP by arsenate-depleted Type 2 mitochondria*

Conditions are as described in Fig. 3. ▲, Atractylate-sensitive ATP binding; ●, atractylate-insensitive binding. $K_D' = 2\mu$M (25% of total binding); $K_D'' = 20\mu$M (75% of total binding); total binding = 40pmol·mg of protein$^{-1}$.

double-reciprocal plots of $v_{80}$ rates of uptake and of binding constants from Scatchard plots of equilibrium values of ATP uptake.

Fig. 7 is a Scatchard plot of the uptake of ATP by arsenate-depleted Type 2 mitochondria. Despite the considerable differences in membrane composition and organization produced by anaerobiosis, the $K_D$ values for atractylate-sensitive binding and the maximal extent of ATP uptake are similar to those of Type 1 mitochondria. $K_m$ and $V_{80,max.}$ values for rates of uptake are also virtually unaltered.

Antibiotic inhibitors of mitochondrial protein synthesis almost completely prevent the formation of particulate mitochondrial cytochromes, and yeast cells are not able to grow on non-fermentable substrates. Thus in the presence of erythromycin cells are grown on glucose medium, which causes catabolite repression of mitochondrial formation (Clark-Walker & Linnane, 1967). Accordingly, the effects of the inhibition of mitochondrial protein synthesis on the adenine nucleotide transporter are assessed by comparing catabolite-repressed Type 3 mitochondria with erythromycin-inhibited Type 4 mitochondria.

Lineweaver–Burk plots of $v_{80}$ rates of ATP uptake by Type 3 and Type 4 mitochondria in the presence and in the absence of atractylate are compared in Fig. 8(*a*) and 8(*b*), and Scatchard plots of the extents of ATP uptake are compared in Figs. 9(*a*) and 9(*b*). Type 3 mitochondria give kinetic and binding parameters for atractylate-sensitive ATP uptake that are very similar to those for Type 1 mitochondria. However, the Scatchard plot for atractylate-insensitive binding by Type 3 mitochondria (Fig. 9*a*) deviates somewhat from a vertical straight line at the high ratios of [bound ATP] to [free ATP], corresponding to the lowest concentrations of added ATP; approx. 20 pmol·mg of protein$^{-1}$ is bound in this way, with a $K_D$ value of 5 $\mu$M.

The uptake of ATP by Type 4 mitochondria is markedly different from that by Type 3 mitochondria. First, Fig. 8(*b*) shows that ATP uptake is totally insensitive to atractylate, and apparently does not follow saturation kinetics, having a $K_m$ value >2 mM. The Scatchard plot of ATP uptake by Type 4 mitochondria (Fig. 9*b*) shows that the plot of atractylate-insensitive uptake deviates considerably from a vertical straight line, and has a saturable component with a $K_D$ value of 20 $\mu$M that binds about 100 pmol of ATP·mg of protein$^{-1}$.

The uptake of ATP by Type 5 mitochondria is also totally insensitive to atractylate (up to 500 $\mu$M), as shown in the Scatchard plot in Fig. 10. A double-reciprocal plot of the concentration-dependence of $v_{80}$ rates of ATP uptake by Type 5 mitochondria is similar to that of Type 4 mitochondria. However, the Scatchard plot of atractylate-insensitive ATP uptake by twice-washed Type 5 mitochondria (Fig. 10*a*) has a large saturable component at low concentrations of ATP, which has a $K_D$ value of 15 $\mu$M and binds 80 pmol·mg of protein$^{-1}$. The saturable atractylate-insensitive component is somewhat diminished by gradient purification of the Type 5 mitochondria, which bind only 50 pmol·mg of protein$^{-1}$ with a $K_D$ value of 15 $\mu$M, suggesting that this

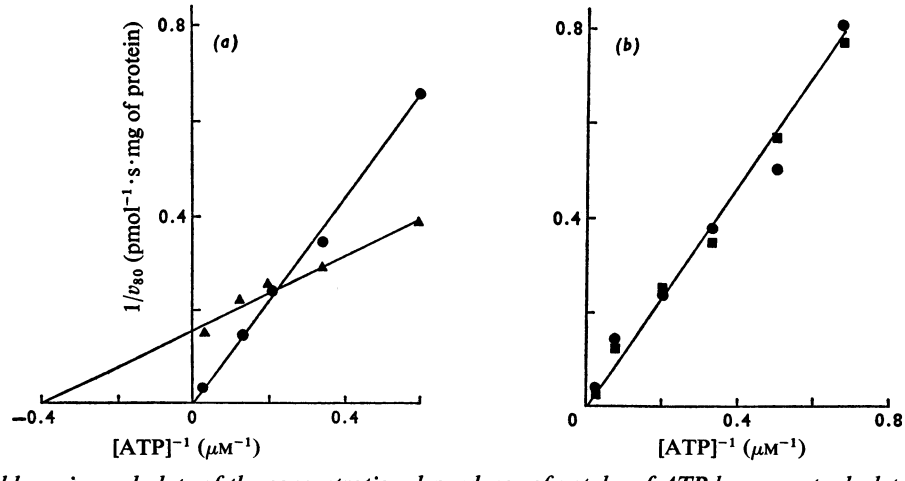



Fig. 8. *Double-reciprocal plots of the concentration-dependence of uptake of ATP by arsenate-depleted Type* 3 (*a*) *and Type* 4 (*b*) *mitochondria*

Conditions are as described in Fig. 2. (*a*) Type 3 mitochondria; (*b*) Type 4 mitochondria. ▲, Atractylate-sensitive ATP uptake; ●, atractylate-insensitive ATP uptake; ■, total ATP uptake. Calculated kinetic constants for atractylate-sensitive ATP uptake: $K_m = 2.5$ $\mu$M, $V_{80,max.} = 7$ pmol·mg of protein$^{-1}$.

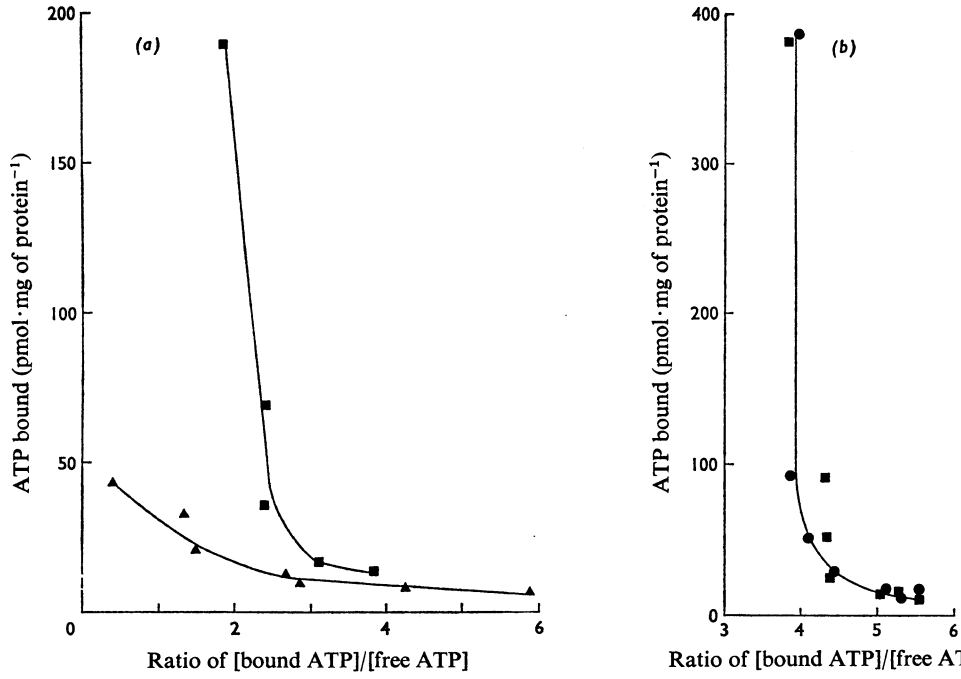



Fig. 9. *Scatchard plots of the uptakes of ATP by arsenate-depleted Type* 3 (*a*) *and Type* 4 (*b*) *mitochondria*

Conditions are as described in Fig. 3. (*a*) Type 3 mitochondria; (*b*) Type 4 mitochondria. ■, Total ATP binding; ▲, atractylate-sensitive ATP binding; ●, ATP binding insensitive to atractylate (0.1 mM). Calculated binding constants for atractylate-sensitive binding by Type 3 mitochondria: $K_D' = 1\,\mu$M (25% of total binding); $K_D'' = 20\,\mu$M (75% of total binding); total binding = 50 pmol·mg of protein$^{-1}$. Calculated binding constants for saturable component of atractylate-insensitive ATP binding: (*a*) Type 3 mitochondria, $K_D = 5\,\mu$M, binding 30 pmol·mg of protein$^{-1}$; (*b*) Type 4 mitochondria, $K_D = 20\,\mu$M, binding 80 pmol·mg of protein$^{-1}$.

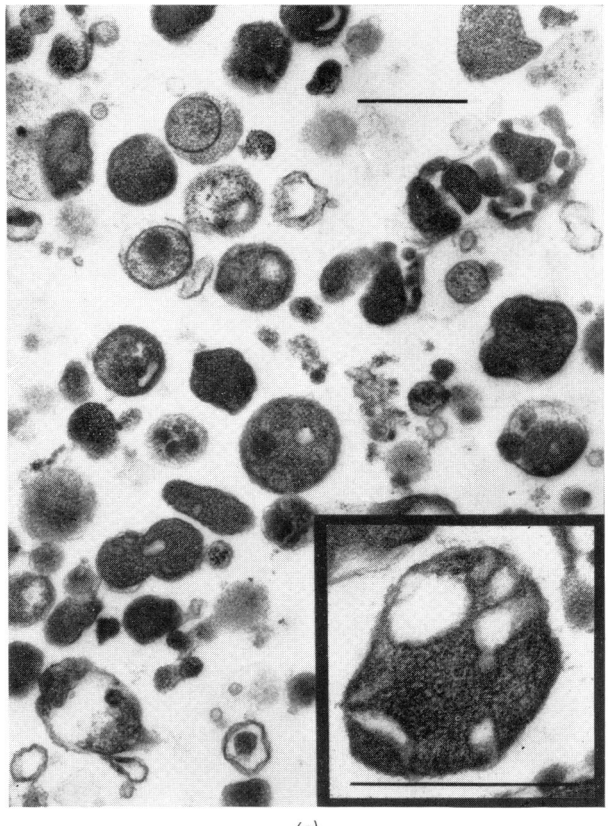
mode of binding is partly due to contamination with non-mitochondrial membranes.

*Criteria of purity and intactness of Type* 4 *and Type* 5 *mitochondria*

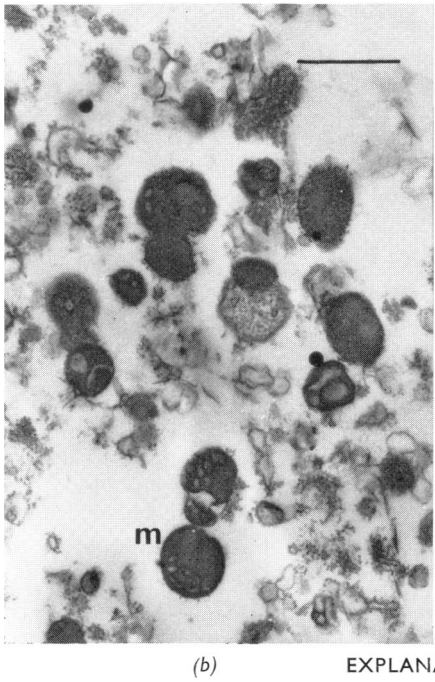
Since Types 4 and 5 mitochondria have markedly altered ATP-binding properties, it is important to establish that these changes are not simply because the organelles are damaged leading to the loss of atractylate-sensitivity, or alternatively are due to contamination with extramitochondrial membranes that bind ATP in an atractylate-insensitive reaction. Electron microscopy of the Types 4 and 5 mitochondria was performed to determine the purity of the organelles and to detect possible gross damage to membranes. The cytochrome *c* content and sucrose-impermeable spaces of the mitochondria were measured to reveal damage to outer and inner mitochondrial membranes respectively.

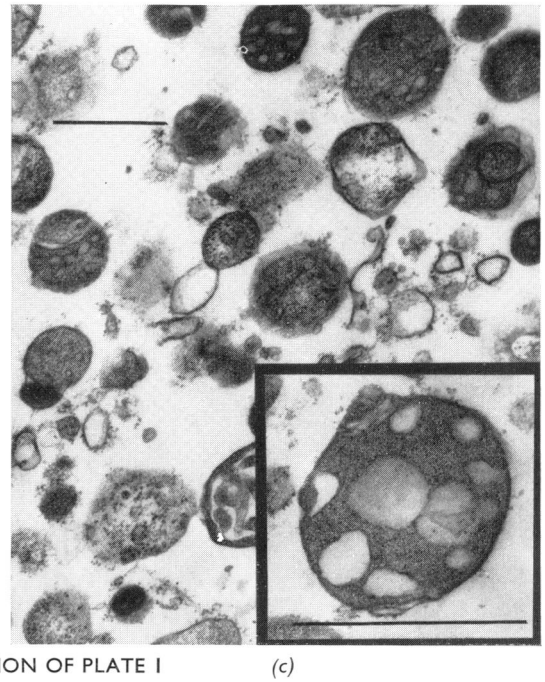
Electron micrographs of twice-washed Type 4 mitochondria are shown in Plate 1(*a*). The low-power field shows that the Type 4 mitochondria are primitive in morphology, owing to the loss of the products of the mitochondrial protein-synthesizing system, but the membranes do not appear to be damaged (see high-magnification inset), and are contaminated only by relatively minor quantities of non-mitochondrial material. Plate 1(*b*) shows that twice-washed Type 5 mitochondria are grossly contaminated with non-mitochondrial material. However, gradient purification of the Type 5 mitochondria, shown in Plate 1(*c*), eliminates most of the non-mitochondrial membrane; the Type 5 mitochondria appear to be intact, and are similar in morphology to Type 4 mitochondria.

Table 3 gives the cytochrome *c* contents of Types 1, 3, 4 and 5 mitochondria, and the sucrose-impermeable spaces of Types 1, 4 and 5 mitochondria. Type 1 mitochondria contain more cytochrome ($c+c_1$) than do Types 3, 4 and 5, as the latter are catabolite repressed owing to growth on glucose medium. Types 4 and 5 mitochondria contain similar amounts of cytochrome *c* to the cytochrome content ($c+c_1$) of



(*a*)



(*b*) (*c*)

EXPLANATION OF PLATE 1

*Electron micrographs of Type* 4 *and Type* 5 *mitochondria*

Mitochondria were isolated and prepared for electron microscopy as described in the Experimental section. (*a*) Twice-washed Type 4 mitochondria. Low-power field, magnification × 25800; high-power inset of single mitochondrion, magnification × 66000. (*b*) Twice-washed Type 5 mitochondria, magnification × 25800, m = mitochondrion. (*c*) Gradient-purified Type 5 mitochondria. Low-power field, magnification × 25800; high-power inset of single mitochondrion, magnification × 60000. The bar represents 0.5 $\mu$m.

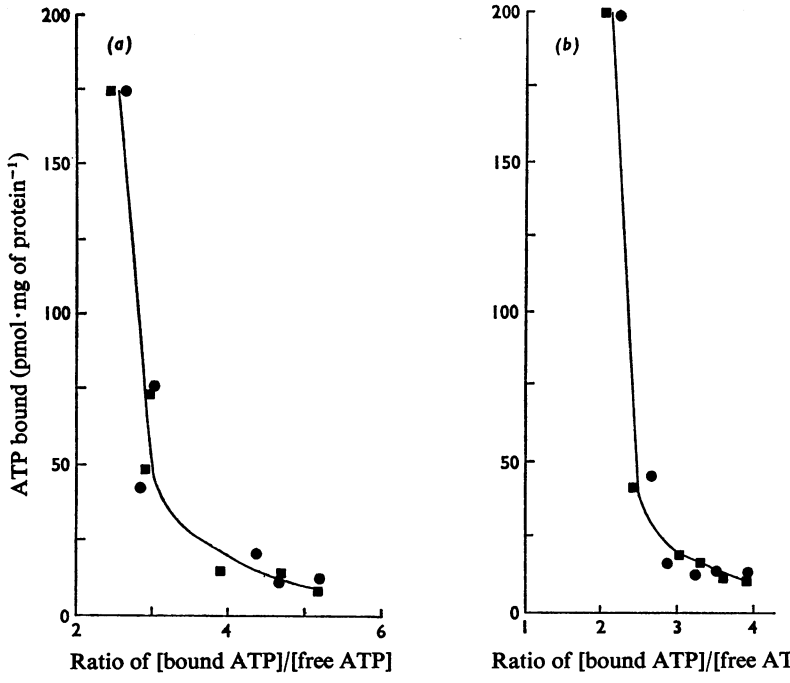



Fig. 10. *Scatchard plots of the uptakes of ATP by arsenate-depleted Type 5 mitochondria before and after gradient purification*

Conditions are as described in Fig. 3. (*a*) Twice-washed Type 5 mitochondria; (*b*) twice-washed mitochondria that were subsequently purified on a discontinuous sorbitol gradient as described in the Experimental section. ●, Atractylate-insensitive binding; ■, total binding. Calculated binding constants for the saturable component of atractylate-insensitive ATP uptake: (*a*) $K_D = 15\,\mu M$, binding = 70pmol·mg of protein$^{-1}$; (*b*) $K_D = 15\,\mu M$, binding = 50pmol·mg of protein$^{-1}$.

Table 3. *Parameters of the intactness of different types of yeast mitochondria*

The cytochrome ($c+c_1$) contents (nmol·mg of protein$^{-1}$) of Types 1 and 3 mitochondria and the cytochrome *c* contents of Types 4 and 5 mitochondria were determined as described in the Experimental section. Mitochondria were preincubated in arsenate, then incubated in the medium used for the assay of ATP uptake plus $^3H_2O$ and [$^{14}$C]sucrose to determine water space, sucrose space and the sucrose-impermeable space ($\mu$l·mg of protein$^{-1}$) as described in the Experimental section.

| Mitochondria | Cytochrome ($c+c_1$) | Water space | Sucrose space | Sucrose-impermeable space |
|---|---|---|---|---|
| Type 1 | 0.8 | 8.6 | 6.6 | 2.0 |
| Type 3 | 0.5 | — | — | — |
| Type 4 | 0.6 | 8.2 | 7.4 | 1.8 |
| Type 5 | 0.4 | 7.6 | 6.0 | 1.6 |

Type 3 mitochondria, indicating that their outer membrane is intact as judged by this criterion. The sucrose-impermeable spaces of Type 4 and Type 5 mitochondria are comparable with that of Type 1 mitochondria, indicating that the inner membranes are intact with respect to permeability function.

## Discussion

### *Parameters of ATP uptake by fully functional yeast mitochondria*

Atractylate-sensitive ATP uptake by arsenate-depleted Type 1 mitochondria has an apparent $K_m$

of 3 $\mu$M, and occurs at high- and low-affinity binding sites in the ratio 1:3 with dissociation constants of 1 and 20 $\mu$M respectively. These results are very similar to those for the atractylate-sensitive uptake of ATP by mammalian mitochondria, but the extent of uptake (50 pmol·mg of protein$^{-1}$) is considerably less than that of mammalian organelles (Klingenberg, 1970). The high activation energies for atractylate-sensitive ATP uptake (+35 and +57 kJ/mol) and the discontinuity in the Arrhenius plot at about 11°C are also properties shared with the adenine nucleotide transporter of mammalian mitochondria (Heldt & Klingenberg, 1968). Neither the kinetics nor the extent of the atractylate-insensitive portion of ATP uptake appear to exhibit saturation, suggesting that the process is non-specific. This concept is also supported by the very low Arrhenius activation energy (6 kJ/mol) for atractylate-insensitive binding. Atractylate inhibits the $P_i$–ATP exchange activity of yeast mitochondria by more than 99% (Groot *et al.*, 1971), indicating that the ATP bound to mitochondria in the presence of atractylate is not available to the adenosine triphosphatase enzyme on the inner surface of the inner mitochondrial membrane. Further, Weidemann *et al.* (1970) report that atractylate-insensitive [$^{14}$C]ADP uptake by mammalian mitochondria is mainly due to the occupation of the sucrose-permeable space of the pellet by free [$^{14}$C]ADP. Thus the non-specific atractylate-insensitive uptake of ADP and ATP probably represents binding to sites outside the mitochondrial matrix.

*Effects of genetic and physiological manipulation on the adenine nucleotide transporter*

Table 4 summarizes the kinetic parameters and binding constants for the uptake of ATP by the five distinct mitochondrial types of *S. cerevisiae*. Both the kinetic parameters and the binding constants for the uptake of ATP by anaerobic mitochondrial precursors (Type 2) and by catabolite-repressed (Type 3) mitochondria are remarkably similar to those of fully functional (Type 1) mitochondria. Since Type 2 mitochondria totally lack the cytochromes and certain of the enzymes of the terminal electron-transport chain, and Type 3 mitochondria have diminished amounts of these enzymes, the number of adenine nucleotide-binding sites in yeast mitochondria is not in direct proportion to the content of respiratory enzymes, in contrast with the apparently fixed ratios reported in mammalian mitochondria (Klingenberg, 1970). However, non-catalytic subunits of inner-membrane enzymes such as cytochrome oxidase are made by anaerobic mitochondria (Schatz *et al.*, 1972), and there may be a stoicheiometry between the transporter proteins and certain of the structural components of the respiratory chain.

The ratio of high-affinity to low-affinity atractylate-

Table 4. *Summary of kinetic and binding parameters for the uptake of ATP by S. cerevisiae*

Results presented elsewhere in this paper are summarized in this table for comparison: (a) arsenate-depleted mitochondria; (b) freshly prepared mitochondria; (c) gradient-purified mitochondria. Atractylate-insensitive uptake does not apparently obey saturation kinetics, and the extent of binding of ATP does not reach a maximal value with increasing concentrations of ATP. However, a portion of atractylate-insensitive ATP binding is saturable in Types 3, 4 and 5 mitochondria.

| Mitochondria ... | (a) | (b) | (a) | (b) | (a) | (a) | (c) |
|---|---|---|---|---|---|---|---|
| Type ... | 1 | 1 | 2 | 3 | 4 | 5 | 5 |
| Atractylate-sensitive uptake | | | | | | | |
| Inhibition by atractylate (%)* | 67 | 60 | 60 | 65 | 0 | 0 | 0 |
| $K_m$ ($\mu$M) | 3 | 3 | 5 | 2.5 | — | — | — |
| $V_{80,max.}$ (pmol·s$^{-1}$·mg of protein$^{-1}$) | 4 | 7 | 8 | 7 | — | — | — |
| $K_D'$† | 1 (25%) | 2 (30%) | 2 (25%) | 2 (25%) | — | — | — |
| $K_D''$† | 20 (75%) | 28 (70%) | 20 (75%) | 20 (75%) | — | — | — |
| Binding sites (pmol·mg of protein$^{-1}$) | 40 | 100 | 50 | 50 | 0 | 0 | 0 |
| Atractylate-insensitive uptake | | | | | | | |
| $K_D$ | — | — | — | 5 | 15 | 15 | 15 |
| Binding sites (pmol·mg of protein$^{-1}$) | — | — | — | 30 | 80 | 70 | 50 |

* Inhibition of the uptake of ATP (2 $\mu$M) by atractylate (100 $\mu$M).

† Values in parentheses give percentages of total atractylate-sensitive uptake.

sensitive ATP-binding sites is constant at approx. 1:3 in Types 1, 2 and 3 mitochondria even though the total amount of bound nucleotide varies. This is also true for ox heart and rat heart mitochondria (Weidemann *et al.*, 1970). The existence of a fixed 1:3 ratio of binding sites in organelles that differ widely in composition and origin argues strongly in favour of a tetramer model for the adenine nucleotide transporter. In this model, originally suggested by Weidemann *et al.* (1970), one of the four sites is on the inner surface of the inner membrane, is fully saturated with the endogenous (ADP+ATP) and hence has a very high apparent affinity for added adenine nucleotides ($K_D' = 1\,\mu M$ for binding ATP). The other three sites on the outside of the inner mitochondrial membrane do not have direct access to endogenous nucleotides, and the affinity of these sites for added nucleotides is lower, but reflects the true binding affinity of the transporter ($K_D'' = 20\,\mu M$ for binding ATP).

Type 4 mitochondria from erythroymcin-inhibited yeast cells and Type 5 mitochondria from $\rho^0$ petite cells (see Perkins *et al.*, 1973) both totally lack the atractylate-sensitive uptake of ATP. Weidemann *et al.* (1970) have shown that if mammalian mitochondria are extensively disrupted by sonication or servere detergent treatment, the uptake of adenine nucleotides largely loses its sensitivity to atractylate, but the binding constants for ADP uptake are not greatly altered. Less-severe detergent treatments result in the formation of sucrose-permeable vesicles that retain the atractylate-sensitive binding of ADP and ATP (Winkler & Lehninger, 1968). We have found that the sonication of Type 1 mitochondria also causes progressive loss of the atractylate-sensitivity of ATP uptake in *S. cerevisiae* mitochondria, but extensive comminution of the organelles is required to eliminate atractylate inhibition totally (M. Perkins & J. M. Haslam, unpublished work). However, both Type 4 and Type 5 mitochondria appear to be intact as judged by electron microscopy, and have normal cytochrome *c* contents and sucrose-impermeable spaces, indicating that the loss of high-affinity atractylate-sensitive ATP uptake by Type 4 and Type 5 mitochondria is not simply due to damage of the organelles.

Scatchard plots of the residual atractylate-insensitive ATP uptake show that both Type 4 and Type 5 mitochondria contain a component with a low affinity for ATP ($K_D = 15\,\mu M$), which binds a similar amount of ATP to the atractylate-sensitive sites of Type 1 mitochondria (80 pmol·mg of protein$^{-1}$). Since this saturable atractylate-insensitive component is not detectable in Type 1 and Type 2 mitochondria, it is suggested that the low-affinity ATP-binding component of Type 1 mitochondria loses its atractylate-sensitivity, but is otherwise retained in Type 4 and Type 5 mitochondria. However, an alternative interpretation is that atractylate-insensitive ATP binding is due to non-mitochondrial contaminants, as Type 3 mitochondria also possess a small amount of a similar component ($K_D = 5\,\mu M$, binding 30 pmol·mg of protein$^{-1}$). The purity of the Type 4 and Type 5 mitochondria was therefore carefully investigated. Electron micrographs show that the Type 4 organelles are very pure, but the twice-washed Type 5 mitochondria are badly contaminated with non-mitochondrial material. However, most of the contamination is eliminated by gradient purification, giving Type 5 organelles that still retain a high degree of saturable atractylate-insensitive ATP binding (50 pmol·mg of protein$^{-1}$). It is concluded that some of the saturable atractylate-insensitive ATP binding is possibly due to non-mitochondrial contamination, particularly in Type 3 and crude Type 5 mitochondria, but most of the atractylate-insensitive component is retained in very pure Type 4 and Type 5 organelles, and probably represents a modified mitochondrial ATP transporter. The presence of a modified transporter in Type 4 and Type 5 mitochondria would, moreover, provide a mechanism for the entry of the moderate amounts of ADP and ATP found in these organelles.

Subik *et al.* (1972) reported that, if ATP uptake by mitochondria is inhibited by bongkrekic acid, the additional inhibition of respiration by antimycin A leads to a mass formation of respiratory-deficient mutants of *S. cerevisiae*. Further, the multiplication of the respiration-deficient mutants in glucose medium is prevented by bongkrekic acid. Subik *et al.* (1972) propose that ATP is required within the mitochondria for normal mitochondrial DNA replication, and also for some unknown mitochondrial function that enables the continued growth of petite cells on glucose. If the latter conclusions are correct, the absence of an adenine nucleotide transporter in Type 4 mitochondria would induce the petite mutation, as Type 4 mitochondria are respiratory deficient, and the lack of ATP uptake by Type 5 mitochondria would prevent the cells from growing on glucose. Since neither of these predictions is experimentally correct, the present results and those of Subik *et al.* (1972) can only be reconciled if Type 4 and Type 5 mitochondria possess a modified but active adenine nucleotide transporter.

### *Role of the mitochondrial protein-synthesizing system in determining the activity of the adenine nucleotide transporter*

The $\rho^0$ petite mutation results in the loss of mitochondrial DNA and its genetic products including mitochondrial rRNA (for review see Linnane *et al.*, 1972), and Type 5 mitochondria lack both mitochondrial ribosomes and their products. Erythromycin inhibits the activity of the mitochondrial

protein-synthesizing system of Type 4 mitochondria. Thus the extensive modification of the mitochondrial adenine nucleotide transporter in both Type 4 and Type 5 mitochondria indicate that at least one of its components, presumably that responsible for binding atractylate, is synthesized by mitochondrial ribosomes. It is also probable that this component of the transporter is coded by mitochondrial DNA, but further proof is required, as a nuclear mRNA species coding for the component could normally be translated by the mitochondrial ribosomes. On the other hand, it is likely that certain of the components of the adenine nucleotide transporter are coded by the nucleus and translated by the cell-sap ribosomes, as some residual low-affinity ATP binding apparently remains in Type 5 mitochondria. Thus the adenine nucleotide-transport system is under a dual genetic control in the same manner as other complex inner-membrane functions of yeast mitochondria such as cytochrome oxidase and adenosine triphosphatase (for review see Linnane *et al.*, 1972).

The present results contradict a report by Kolarov *et al.* (1972*a*), that mitochondria from a respiratory-deficient strain of *S. cerevisiae* retain a high-affinity adenine nucleotide transporter. However, the results of Kolarov *et al.* (1972*a*) are open to criticism, as the atractylate-sensitivity of their petite mitochondria is not documented, and the mitochondria were extensively damaged as judged by sucrose-impermeable space and electron microscopy, leading to the possibility that they were measuring a modified form of the saturable atractylate-insensitive low-affinity binding site observed by us in petite mitochondria. Although the respiratory-deficient strain of Kolarov *et al.* (1972*a*) is not genetically characterized, it could be a cytoplasmic mutant that retains mitochondrial DNA ($\rho^-$) and possibly possesses some mitochondrial genetic information, or a mutant that has no detectable mitochondrial DNA ($\rho^0$) and consequently lacks mitochondrial genetic information (Linnane *et al.*, 1972). It is unlikely that this is responsible for the difference between the two sets of results, because our results would predict that all $\rho^0$ petite strains and those $\rho^-$ strains that lack active mitochondrial ribosomes would also lack an atractylate-sensitive mitochondrial ATP transporter. So far all $\rho^-$ strains that have been investigated lack mitochondrial protein synthesis (Kuzela & Grecna, 1969; Schatz & Salzgaber, 1969; Kellerman *et al.*, 1971).

Kolarov *et al.* (1972*b*) have reported that the lesion in the oxidative phosphorylation $op_1$ mutant is a change in a nuclear gene that results in altered properties of the adenine nucleotide transporter, notably a two- to four-fold decrease in the translocation rate of ADP. Thus both nuclear and mitochondrial genes are implicated in the function of the mitochondrial adenine nucleotide transporter. However, it is not possible to say whether the $op_1$ mutation or the $\rho^0$ mutation directly affect the catalytic components of the transporter, as a change in a nuclear or mitochondrially coded membrane protein could produce all the effects so far observed via a reorganization of the membrane environment of the transporter. The selection of other nuclear and mitochondrial mutants that affect the adenine nucleotide transporter may eventually lead to the characterization of the components of this mitochondrial transport system and the identification of their sites of synthesis.

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