Biochem. J. (1973) **134**, 923–934
Printed in Great Britain

Biogenesis of Mitochondria*

THE EFFECTS OF PHYSIOLOGICAL AND GENETIC MANIPULATION OF *SACCHAROMYCES CEREVISIAE* ON THE MITOCHONDRIAL TRANSPORT SYSTEMS FOR TRICARBOXYLATE-CYCLE ANIONS

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(*Received* 15 *February* 1973)

1. Kinetic and equilibrium parameters for the uptake of L-malate, succinate, citrate and α-oxoglutarate by fully functional mitochondria of *Saccharomyces cerevisiae* were determined. 2. The uptake of L-malate and succinate is mediated by a common carrier, and two other distinct carriers mediate the uptake of citrate and α-oxoglutarate. 3. The properties of the carrier systems for L-malate, succinate and citrate closely resemble those of mammalian mitochondria, but the α-oxoglutarate carrier differs from the mammalian system in minor respects. 4. The composition of the yeast mitochondria was extensively manipulated by (a) anaerobiosis, (b) catabolite repression, (c) inhibition of mitochondrial protein synthesis and (d) elimination of mitochondrial DNA by mutation. 5. The carrier systems for L-malate, succinate, citrate and α-oxoglutarate are essentially similar in the five different types of mitochondria. 6. It is concluded that all the protein components of the carrier systems for L-malate, succinate, citrate and α-oxoglutarate are coded by nuclear genes and synthesized extramitochondrially by cell-sap ribosomes.

Mammalian mitochondria have been shown to contain at least seven specific carrier systems that facilitate the transport of anions across the inner mitochondrial membrane (Chappell, 1968; Chappell & Robinson, 1968; Klingenberg, 1970). The most important properties that establish the presence of a biological transporter for an anion are (i) saturation kinetics for the concentration-dependence of the rate of uptake, (ii) existence of specific inhibitors and (iii) substrate specificity. The anion transporters of mitochondria are not obligatorily linked to the expenditure of energy, but facilitate the exchange-diffusion of specific anions, e.g. the dicarboxylate transporter exchanges dicarboxylate anions for other dicarboxylate anions or phosphate, and the tricarboxylate transporter exchanges tricarboxylate anions for other tricarboxylate or dicarboxylate anions (Chappell, 1968; Chappell & Robinson, 1968; Klingenberg, 1970).

Direct attempts to isolate the mammalian mitochondrial transporter proteins have been unsuccessful (Chavin, 1971). However, partial characterization of the proteins of certain bacterial sugar-transport systems has been achieved by the use of physiological and genetic manipulation (for reviews see, Kaback, 1970; Chavin, 1971). In the present work a similar approach is initiated with 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 far-reaching physiological control, and both nuclear and mitochondrial mutants affecting mitochondrial components are readily selected.

In contrast with the detailed investigations of mammalian mitochondrial transport systems (Klingenberg, 1970), very little is known of the anion-transport systems of yeast mitochondria. Kolarov *et al.* (1972) have used the indirect method of swelling mitochondria in ammonium salts to demonstrate the presence of carriers for phosphate, succinate and L-malate in fully functional, anaerobic and petite mitochondria. In the present study anion transport is measured by a direct method to obtain parameters of the rate and extent of uptake of four tricarboxylate-cycle intermediates, succinate, L-malate, citrate and α-oxoglutarate. The effects of a number of physiological and genetic manipulations on the properties of the anion-transport systems are investigated. These manipulations include catabolite repression, anaerobiosis, inhibition of the mitochondrial protein-synthesizing system by erythromycin and elimination of mitochondrial DNA by mutation. A companion paper reports similar studies on the adenine nucleotide transporter of yeast mitochondria (Haslam *et al.*, 1973). A preliminary report of some of this work has been published elsewhere (Perkins *et al.*, 1972).

* This paper is no. 31 in the series. No. 30 is Howell *et al.* (1973).

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Experimental

Yeast strains

Two haploid strains of *S. cerevisiae* were used; the respiratory-competent strain, L 410 (*α his ura*), and the respiratory-deficient petite strain E 5, which contains no mitochondrial DNA and is designated ρ^0 and was obtained from strain L 410 by ethidium bromide treatment (Nagley & Linnane, 1970).

Growth conditions

Cells were grown at 28°C in a 1% Difco yeast extract–*Saccharomyces* salts medium (Wallace *et al.*, 1968) plus the carbon substrates and lipid supplements indicated below. Five distinct cell types were grown, and the mitochondria isolated from each cell type are referred to as Types 1–5. References to the properties of the different types of mitochondria are found in the reviews by Linnane & Haslam (1970) and Linnane *et al.* (1972). Cells were grown as follows.

Type 1: strain L 410 grown aerobically in 1% (w/v) ethanol medium. These cells contain de-repressed fully functional mitochondria that exhibit good respiratory control and high oxidase activities. The properties of Type 1 mitochondria are very similar to those of mammalian mitochondria, and are compared in the present paper with the properties of the remaining four modified mitochondrial species, (Types 2–5) (Ephrussi, 1953).

Type 2: strain L 410 grown anaerobically on galactose (4%, w/v) medium supplemented with ergosterol (20mg/l), and with Tween 80 (0.5%, w/v) as a source of unsaturated fatty acids. Type 2 mitochondria totally lack mitochondrial cytochromes and the capacity for cyanide- and antimycin A-sensitive respiration or phosphorylation, but retain a folded inner membrane and an active mitochondrial protein-synthesizing system (Watson *et al.*, 1970; Davey *et al.*, 1969).

Type 3: strain L 410 grown aerobically on glucose (2%, w/v) medium. Type 3 mitochondria are catabolite repressed, have lower contents of particulate cytochromes, and have a less-developed inner membrane than Type 1 mitochondria (Ephrussi, 1953; Lukins *et al.*, 1968).

Type 4: strain L 410 grown aerobically on glucose (2%, w/v) plus erythromycin (1mg/ml) to inhibit mitochondrial protein synthesis. Type 4 mitochondria are catabolite repressed owing to growth on glucose, possess mitochondrial DNA and the mitochondrial protein-synthesizing system, but otherwise phenotypically resemble petite Type 5 mitochondria (Davey *et al.*, 1969; Clark-Walker & Linnane, 1967).

Type 5: strain E5 grown aerobically on glucose (4%, w/v). Type 5 (cytoplasmic petite) mitochondria are catabolite repressed owing to growth on glucose, lack detectable mitochondrial DNA, mitochondrial rRNA, the particulate cytochromes (c_1, a and a_3) and respiratory activity, but nevertheless have a primitive inner-membrane system (Nagley & Linnane, 1970; Perlman & Mahler, 1970; Wintersberger, 1967).

Preparation of mitochondria

Types 1–5 mitochondria were prepared from protoplasts of Types 1–5 cells as described previously (Watson *et al.*, 1970). The crude mitochondrial pellet was washed by centrifuging twice in sorbitol (0.5M), ethanedioxybis(ethylamine)tetra-acetate (EGTA; 1mM), bovine serum albumin (2mg/ml) (Commonwealth Serum Laboratories, Parkville, Vic., Australia), 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonate (10mM), final pH6.5.

Measurement of uptake of ^{14}C-labelled anions

Mitochondria (0.5–1.0mg of protein) were 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), ethanedioxybis(ethylamine)tetra-acetate (1mM), KH_2PO_4 (2mM), final pH6.5. Antimycin A (1μg/ml) was added to inhibit completely the oxidation of substrates, and consequently the mitochondria are in a non-energized state. The following ^{14}C-labelled anions were used: L-[U-^{14}C]malate, [2,3-^{14}C]succinate, α-[5-^{14}C]oxoglutarate, [1,5-^{14}C]-citrate; the initial specific radioactivities of the anions were 5–25mCi/mmol and appropriate dilutions were made by adding a solution of the highest commercial grade available of the appropriate anion, adjusted to pH6.5. Inhibitors were added 2min before the addition of ^{14}C-labelled substrates (0.1μCi) and stopped by filtering the medium through a Millipore filter (0.45μm pore size) under reduced pressure in less than 2s. The mitochondria were then washed on the filter with 6ml of ice-cold incubation medium. The filters were dried and counted for radioactivity in the Picker Nuclear-Liquimat 220 counter in 5ml of toluene scintillator fluid containing 2,5-diphenyloxazole (5%; Calbiochem, San Diego, Calif., U.S.A.) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.3%; Calbiochem). The shortest incubation possible by this method was approx. 5s; incubations of 10, 20 and 60s were also performed, and maximal uptake was observed after 20s for all substrates. It was not possible to determine initial rates of uptake, as even at the shortest incubation time uptake proceeds more than 50% towards equilibrium. To compare the kinetics of anion uptake in the different types of mitochondria we have defined an empirical reaction rate v_{80}, which is 80% of the extent of anion uptake divided by the time taken to attain this extent. v_{80} values are obtained from the progress curve of anion uptake. Since the concentration of anions in the medium does not decrease by more than 2% in any

experiment, values may be used to compare the properties of different types of mitochondria. Similarly, empirical K_m and $V_{80,max.}$ values for the rates of uptake of anions by the five types of mitochondria are calculated from double-reciprocal plots of v_{80} values at five different concentrations of anion in the range 0.5–15mM. Empirical Michaelis constants (K_m) and maximal extents of anion uptake ($V_{max.}$) were determined from double-reciprocal plots of the equilibrium extents of uptake and external anion concentrations.

In earlier experiments it was not appreciated that the Millipore filters adsorb variable amounts of ^{14}C-labelled tricarboxylate-cycle anions and rates of uptake, particularly of succinate, were overestimated (Perkins *et al.*, 1972). In the present experiments Millipore filters were preincubated with 10mM-anion for 2h and washed with water before use. This treatment prevents the subsequent adsorption of ^{14}C-labelled anions by the filters.

Determination of sucrose-impermeable space

Mitochondria (about 20mg of protein) were incubated for 20min at 30°C in 0.4ml of the anion-uptake incubation medium plus ^3H_2O (0.1μCi) and [^{14}C]sucrose (0.02μCi). The mitochondria were centrifuged in a graduated calibrated tube and the packed volume of mitochondria was measured. The supernatant was carefully removed, and the ^3H and ^{14}C radioactivity of 50μl portions counted. The pellet was dissolved in an equal volume of 1M-NaOH containing sodium deoxycholate (5%, w/v). The protein concentration of a portion of the dissolved pellet was assayed and a second 0.4ml sample was mixed with 0.1ml of 50% trichloroacetic acid to precipitate protein. The protein precipitate was removed by centrifugation and the ^3H and ^{14}C radioactivity of 50μl portions of the supernatant counted in the Picker Nuclear-Liquimat 220 counter in 5ml of the aqueous scintillator fluid used by Clark-Walker & Linnane (1967). From these results the concentrations of ^3H_2O and [^{14}C]sucrose·mg of protein^{-1} were calculated for the initial mitochondrial pellet. The concentrations of ^3H_2O and [^{14}C]sucrose in the initial supernatant then allow the calculation of the volume of mitochondrial water associated with the pellet and the fraction that is penetrated by sucrose. The sucrose-impermeable space is the difference between these two values and represents the matrix volume bounded by intact inner mitochondrial membranes (Amoore & Bartley, 1958; Pfaff, 1967). Protein was assayed as described by Gornall *et al.* (1949).

Reagents

[^{14}C]Succinate, L-[^{14}C]malate, [^{14}C]citrate, α-oxo-[^{14}C]glutarate, [^{14}C]sucrose and ^3H_2O were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Antimycin A, L-malate, citrate, D-isocitrate, α-oxoglutarate and glutarate were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonate and DL-*threo*-β-hydroxyaspartate were from Calbiochem. DL-2-Methylsuccinate was from Eastman–Kodak, Rochester, N.Y., U.S.A. The diethyl esters of *n*-butylmalonate and benzylmalonate were from Koch–Light, Colnbrook, Bucks., U.K. and were converted into the free acids by alkaline hydrolysis.

Results

Uptake of L-malate by fully functional yeast mitochondria

The time-dependence of the uptake of different concentrations of L-[^{14}C]malate by fully functional Type 1 mitochondria is shown in Fig. 1. The uptake of L-malate at external concentrations in the range 0.25–15mM is very rapid, exceeding 60% of the equilibrium extent by the earliest determinable incubation

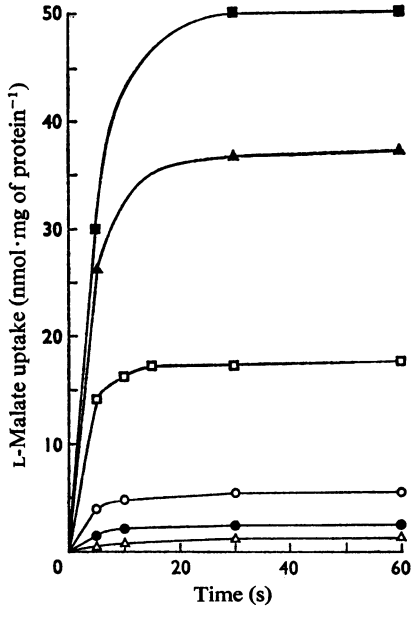

Fig. 1. *Time-dependence of the uptake of different concentrations of* L-[^{14}C]*malate by Type* 1 *mitochondria*

The uptake of L-[^{14}C]malate was determined as described in the Experimental section. The concentrations of L-[^{14}C]malate added to the incubation medium were as follows: △, 0.25mM; ●, 1.0mM; ○, 2.5mM; □, 5mM; ▲, 10mM; ■, 15mM.

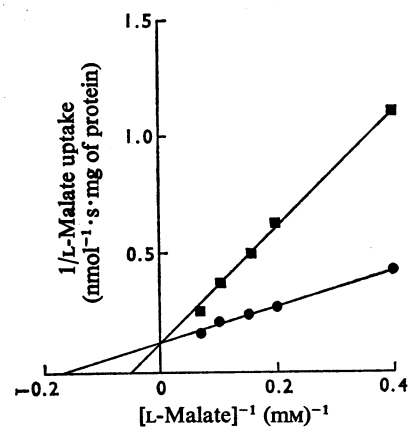

Fig. 2. *Concentration-dependence of the rate of uptake of* L-[^{14}C]*malate by Type* 1 *mitochondria*

v_{80} rates of uptake of L-[^{14}C]malate were determined as described in the Experimental section. The results of a typical experiment are shown in the form of a double-reciprocal plot of the v_{80} rates of uptake against L-malate concentrations; ●, no further additions; ■, 2-*n*-butylmalonate (4mM) added. Calculated kinetic parameters are: K_m 6.1mM; $V_{80,max.}$ 8nmol·s^{-1}·mg of protein^{-1}; K_i for 2-*n*-butylmalonate 1.7mM. In further individual experiments K_m values of 5.3, 6.3 and 7.5mM, and corresponding $V_{80,max.}$ values of 5, 6 and 7nmol·s^{-1}·mg of protein^{-1} were obtained.

time of 5s and reaching equilibrium by 30s. Consequently, initial rates of anion uptake are not determinable, but empirical kinetic parameters for the approach to equilibrium are obtained from the v_{80} rates of uptake defined in the Experimental section. Fig. 2 shows that double-reciprocal plots of the v_{80} rates of uptake and L-malate concentrations in the presence and in the absence of 2-*n*-butylmalonate approximate to straight lines, from which an apparent K_m value of 6.1mM and a $V_{80,max.}$ value of 8nmol·s^{-1}·mg of protein^{-1} are calculated. The analogue 2-*n*-butylmalonate acts as a competitive inhibitor of L-malate uptake with K_i 1.7mM. The values of the kinetic constants vary by up to 30% in individual preparations of Type 1 mitochondria, but are sufficiently reproducible to allow the detection of large changes in the kinetic properties of the transport systems for the five different types of yeast mitochondria used in the present investigation.

Fig. 3 shows the effects of phosphate, 2-*n*-butylmalonate and 2-methylsuccinate on the rate and extent of L-[^{14}C]malate uptake by Type 1 mitochondria. The two analogues inhibit not only the rate

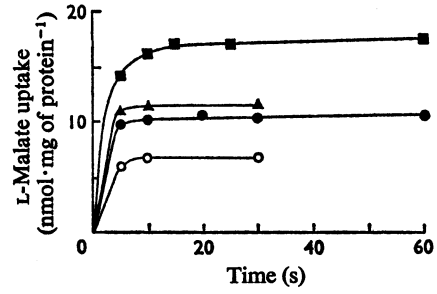

Fig. 3. *Effects of substrate analogues and phosphate on the uptake of* L-[^{14}C]*malate by Type* 1 *mitochondria*

The time-dependence of the uptake of L-[^{14}C]malate (5mM) was determined as described in Fig. 1. The composition of the incubation media was as given in the Experimental section, but with the following variations: ■, control; ▲, 2-methylsuccinate (6mM) added; ●, phosphate omitted; ○, 2-*n*-butylmalonate (6mM) added.

but also the extent of uptake of L-malate at equilibrium. Phosphate stimulates the rate of L-malate uptake as previously reported for mammalian mitochondria (Chappell, 1968), but also unexpectedly stimulates the extent of uptake of the anion at equilibrium. Since phosphate is known to cause swelling of the mitochondrial matrix under certain conditions, the sucrose-impermeable space of Type 1 mitochondria in the presence and in the absence of phosphate was determined under incubation conditions identical with those used for the measurement of L-[^{14}C]malate uptake. Values of 2.8 and 1.6μl·mg of protein^{-1} were obtained respectively in the presence and in the absence of phosphate (2mM). Assuming that the L-malate is accumulated within the sucrose-impermeable space, as suggested by other authors (Pfaff, 1967; van Dam & Tsou, 1968), the values for the sucrose-impermeable space together with the results in Fig. 3 allow the calculation of intramitochondrial substrate concentrations. At an external L-malate concentration of 5mM the intramitochondrial concentration of L-malate is 6.2mM in the presence of phosphate (2mM) and 6.0mM in the absence of phosphate. Thus the stimulation of the extent of L-malate uptake by phosphate is due to the expansion of the mitochondrial matrix. The ratio of intramitochondrial to extramitochondrial concentration of L-malate is 1.2 in both the presence and the absence of phosphate, even though the mitochondria are non-energized owing to the inhibition of respiration by antimycin A. The dependence of the equilibrium extent of L-malate uptake on external anion concentration is shown in Fig. 4. The plot is a straight line from which are

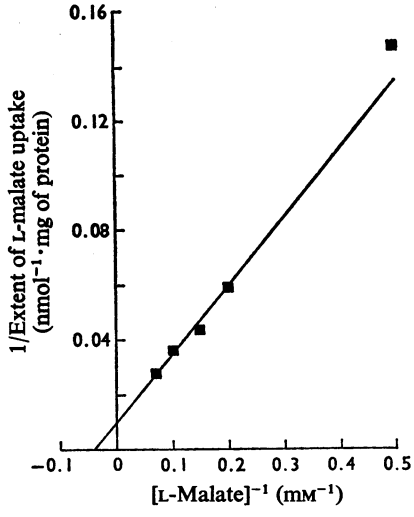

Fig. 4. *Dependence of the extent of uptake of* L-[^{14}C]*-malate by Type* 1 *mitochondria on the external concentration of* L*-malate*

The extent of uptake of L-[^{14}C]malate at equilibrium, determined as described in Fig. 1, was plotted against the external concentration of L-malate in the form of a double-reciprocal plot. The calculated parameters of uptake are: maximal extent of uptake = 100nmol·mg of protein^{-1}; K_m 25mM.

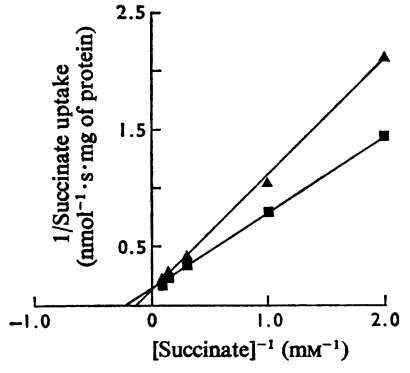

Fig. 5. *Concentration-dependence of the rate of uptake of* [^{14}C]*succinate by Type* 1 *mitochondria*

Rates of uptake of [^{14}C]succinate were determined as described in the Experimental section, and are presented in the form of a double-reciprocal plot of v_{80} rates against the concentrations of succinate. ■, Control; ▲, 2-methylsuccinate (4mM) added. Calculated kinetic parameters are: K_m = 5.6mM; $V_{80,max.}$ = 9nmol·s^{-1}·mg of protein^{-1}; K_i for 2-methylsuccinate = 5mM.

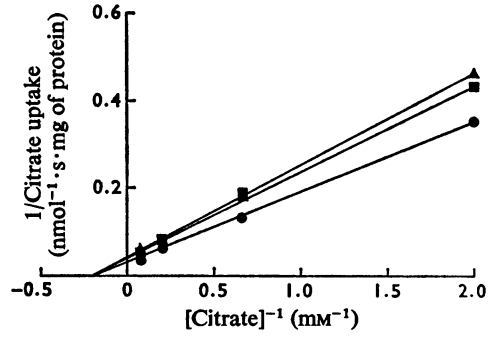

Fig. 6. *Concentration-dependence of the rate of uptake of* [^{14}C]*citrate by Type* 1 *mitochondria*

The uptake of [^{14}C]citrate was measured as described in the Experimental section, and is presented as a double-reciprocal plot of v_{80} rates of uptake against citrate concentrations. Further additions were: ■, none; ●, L-malate (0.1mM); ▲, 2-*n*-butylmalonate (6mM) plus L-malate (0.1mM). Calculated kinetic parameters are: K_m = 5.0mM; $V_{80,max.}$ = 25nmol·s^{-1}·mg of protein^{-1}. In the presence of L-malate $V_{80,max.}$ increases to 35nmol·s^{-1}·mg of protein^{-1}, but the stimulatory effect of L-malate is totally abolished by 2-*n*-butylmalonate (6mM).

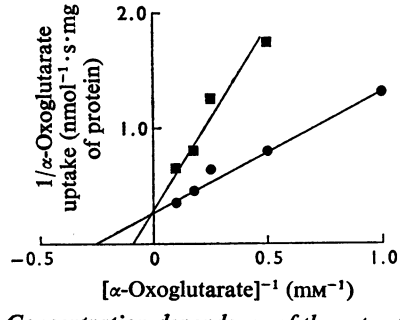

Fig. 7. *Concentration-dependence of the rate of uptake of* α*-oxo*[^{14}C]*glutarate by Type* 1 *mitochondria*

The uptake of α-oxo[^{14}C]glutarate was measured as described in the Experimental section, and is presened as a double-reciprocal plot of v_{80} rates of uptake against α-oxoglutarate concentration. Further additions were: ●, none; ■, DL-*threo*-β-hydroxyaspartate (4mM). Calculated kinetic parameters are: K_m = 4mM; $V_{80,max.}$ = 4nmol·s^{-1}·mg of protein^{-1}; K_i for DL-*threo*-β-hydroxyasparate = 2.3mM.

calculated an apparent K_m value of 25mM and a maximal extent of uptake of 100nmol·mg of protein^{-1} (equal to a intramitochondrial concentration of 36mM).

Uptakes of succinate, citrate and α-oxoglutarate by fully functional yeast mitochondria

The uptakes of succinate, citrate and α-oxoglutarate by Type 1 mitochondria have very similar time-dependences to that of L-malate. Determinations of initial rates of uptake are not possible, but reproducible v_{80} rates are obtained enabling the calculation of empirical K_m and $V_{80,max.}$ values. Typical double-reciprocal plots of the concentration-dependence of the rates of uptake of [^{14}C]succinate, [^{14}C]citrate and α-oxo[^{14}C]glutarate are shown in Figs. 5, 6 and 7 respectively. The kinetic parameters for the uptake of four tricarboxylate-cycle-substrate anions by Type 1 mitochondria, and the effects of substrate analogues and phosphate are summarized in Table 1. The uptake of L-malate is competitively inhibited by 2-*n*-butylmalonate. Uptake is also inhibited by 2-benzylmalonate, malonate, succinate and 2-methylsuccinate, and by the omission of phosphate. The rate of succinate uptake is competitively inhibited by 2-methylsuccinate; inhibition is also caused by 2-*n*-butylmalonate, L-malate and malonate, and by the omission of phosphate. The rate of citrate uptake is inhibited by isocitrate and by the absence of phosphate, but stimulated by L-malate even in the absence of phosphate; the stimulation by L-malate is reflected in an increased $V_{80,max.}$ but an unaltered K_m, and is abolished by 2-*n*-butylmalonate. The rate of uptake of α-oxoglutarate is inhibited by DL-*threo*-β-hydroxyaspartate, which has no effect on the uptakes of L-malate, succinate and citrate.

Table 2 summarizes the equilibrium parameters for the maximal extents of uptake of L-malate, succinate, α-oxoglutarate and citrate by Type 1 mitochondria. The K_m values for the extents of uptake are much higher than the K_m values for the rates of uptake of

Table 1. *Summary of the kinetic parameters for the uptake of tricarboxylate-cycle anions by Type* 1 *mitochondria*

The uptake of ^{14}C-labelled tricarboxylate-cycle anions was measured as described in the Experimental section. v_{80} rates, K_m and $V_{80,max.}$ values were determined graphically as shown in Figs. 1 and 2. K_m and K_i are expressed in mM, and $V_{80,max.}$ and v_{80} in nmol·s^{-1}·mg of protein^{-1}. v_{80} values were determined at the concentrations of substrates and inhibitors given in parentheses in the first and second columns of the table.

		Kinetic parameters			
Substrate anion	Additions to medium	K_m	$V_{80,max.}$	K_i	v_{80}
L-Malate (5.0mM)	None	6.3	8.0	—	3.4
	2-*n*-Butylmalonate (4mM)	—	8.0	1.7	1.6
	2-*n*-Benzylmalonate (4mM)				1.8
	Malonate (4mM)				1.9
	Succinate (4mM)				2.2
	2-Methylsuccinate (4mM)				2.2
	DL-*threo*-β-Hydroxyaspartate (4mM)				3.4
	Phosphate omitted				2.0
Succinate (3.8mM)	None	5.6	9.0	—	3.4
	2-Methylsuccinate (4mM)	—	9.0	5.0	2.2
	2-*n*-Butylmalonate (4mM)				2.2
	L-Malate (4mM)				2.2
	DL-*threo*-β-Hydroxyaspartate (4mM)				3.4
	Phosphate omitted				1.9
Citrate (5.0mM)	None	5.0	25	—	12.8
	L-Malate (0.1mM)	5.0	35	—	15.3
	L-Malate (0.1mM)+2-*n*-butylmalonate (4mM)	—	26	—	12.8
	Phosphate omitted				12.0
	L-Malate (0.1mM), phosphate omitted				14.5
	Isocitrate (4mM)				9.9
	DL-*threo*-β-Hydroxyaspartate (4mM)				12.8
α-Oxoglutarate (3.0mM)	None	4.0	4.5	—	2.0
	DL-*threo*-β-Hydroxyaspartate (4mM)	—	4.5	2.3	0.9
	Aspartate (4mM)				2.0
	Phosphate omitted				1.5
	Glutarate (4mM)				1.1

the four tricarboxylate-cycle anions. The maximal extents of uptake of L-malate, succinate and α-oxoglutarate are very similar, being about 100nmol·mg of protein^{-1}, which corresponds to a maximum intramitochondrial concentration of about 40mM. However, citrate accumulation does not appear to obey simple saturation kinetics, and maximal extents of uptake are very much higher (>250nmol·mg of protein^{-1}, corresponding to an intramitochondrial concentration of >90mM).

Uptake of tricarboxylate-cycle anions by different types of yeast mitochondria

The uptakes of L-malate, succinate, citrate and α-oxoglutarate by anaerobic (Type 2), catabolite-repressed (Type 3), erythromycin-inhibited (Type 4) and petite (Type 5) mitochondria follow essentially the same kinetics as in fully functional (Type 1) mitochondria. Accordingly, v_{80} rates of uptake were measured and empirical kinetic constants were calculated.

Table 3 summarizes the kinetic parameters of anion uptake by Type 2 mitochondria. The transport systems for L-malate, succinate, α-oxoglutarate and citrate are present in the anaerobic mitochondrial precursor structures, and have essentially the same basic properties as those of Type 1 mitochondria. However, there are minor changes in some kinetic parameters; the K_m values for L-malate, citrate and α-oxoglutarate uptake are higher, the $V_{80,max.}$ values for L-malate and succinate uptake decrease, and the $V_{80,max.}$ values for citrate and α-oxoglutarate increase compared with the values for Type 1 mitochondria. All four carrier systems are affected by characteristic inhibitors and activators in the same way as Type 1 mitochondria.

The kinetic parameters of the anion-transport systems of Type 3 and Type 4 mitochondria are compared in Table 4. Although Type 4 mitochondria lack the products of the mitochondrial protein-synthesizing system owing to growth in the presence of erythromycin, their anion-transport systems have

Table 2. *Equilibrium parameters for the extents of uptake of tricarboxylate-cycle anions by Type 1 mitochondria*

K_m values (mM) and maximum extent ($S_{max.}$) values for the uptake of ^{14}C-labelled anions were determined as described in Fig. 4 in the presence of phosphate. $S_{max.}$ values are expressed (a) in nmol·mg of protein^{-1}, (b) as an intramitochondrial concentration (mM) calculated by assuming that the anions are accumulated in the sucrose-impermeable space of 2.8μl·mg of protein^{-1}.

	Equilibrium parameters		
		$S_{max.}$	
Substrate anion	K_m	(a)	(b)
L-Malate	25	100	36
Succinate	12	100	36
α-Oxoglutarate	30	120	43
Citrate	>20	>250	>90

Table 3. *Summary of the kinetic parameters for the uptake of tricarboxylate-cycle anions by Type 2 mitochondria*

Methods were as in Table 1.

		Kinetic parameters			
Substrate anion	Additions to medium	K_m	$V_{80,max.}$	K_i	v_{80}
L-Malate (5.0mM)	None	12	4.0	—	1.9
	2-*n*-Butylmalonate (7.5mM)	—	4.0	4.2	1.0
	2-Methylsuccinate (4mM)				1.2
	Phosphate omitted				1.3
Succinate (7.5mM)	None	4.6	4.6	—	2.5
	2-Methylsuccinate (4mM)	—	4.6	7.2	1.2
	2-*n*-Butylmalonate (4mM)				1.7
	Phosphate omitted				1.7
Citrate (7.5mM)	None	8.3	48	—	23
	L-Malate (0.1mM)				33
	L-Malate (0.1mM)+2-*n*-butylmalonate (4mM)				24
	Phosphate omitted				24
α-Oxoglutarate (5.0mM)	None	16.0	10.0	—	3.7
	DL-*threo*-β-Hydroxyaspartate (8mM)				2.0
	Glutarate (4mM)				3.2

Table 4. *Summary of the kinetic parameters for the uptake of tricarboxylate-cycle anions by Type 3 and Type 4 mitochondria*

Methods were as in Table 1.

		Kinetic parameters							
		Type 3 mitochondria				Type 4 mitochondria			
Substrate anion	Additions to medium	K_m	$V_{80,max.}$	K_i	v_{80}	K_m	$V_{80,max.}$	K_i	v_{80}
L-Malate (5.0mM)	None	11.0	9.0	—	2.9	11.5	9.5	—	2.7
	2-*n*-Butylmalonate (4mM)	—	9.0	3.5	1.5	—	9.0	3.7	1.1
	2-Methylsuccinate (4mM)				2.3				1.4
	Phosphate omitted				2.1				2.3
Succinate (7.5mM)	None	5.4	7.2	—	5.2	5.4	6.7	—	5.0
	2-Methylsuccinate (4mM)	—	7.2	10.0	3.6	—	6.7	8.6	3.1
	2-*n*-Butylmalonate (4mM)				2.7				3.6
	Phosphate omitted				3.9				4.8
Citrate (5.0mM)	None	8.0	46	—	19	8.0	50	—	20
	L-Malate (0.1mM)	8.0	57	—	22	8.0	64	—	28
	L-Malate (0.1mM)+2-*n*-butylmalonate (4mM)		46	—	19				23
α-Oxoglutarate (3mM)	None	10.0	4.0	—	1.2	10.0	4.2	—	1.5
	DL-*threo*-β-Hydroxyaspartate (4mM)				0.8				0.9

Table 5. *Summary of the kinetic parameters for the uptake of tricarboxylate-cycle anions by Type 5 mitochondria*

Methods were as in Table 1.

Substrate anion	Additions to medium	Kinetic parameters K_m	$V_{80,max.}$	K_i	v_{80}
L-Malate (5mM)	None	9.5	8.3	—	3.5
	2-*n*-Butylmalonate (4mM)			3.1	1.6
	2-Methylsuccinate (4mM)				2.3
	Phosphate omitted				2.9
Succinate (3.8mM)	None	4.5	6.0	—	3.1
	2-*n*-Butylmalonate (4mM)				
	2-Methylsuccinate (4mM)				1.6
	Phosphate omitted				1.9
Citrate (4.0mM)	None	7.1	36	—	11.6
	L-Malate (0.1mM)		44	—	14.3
	L-Malate (0.1mM)+2-*n*-butylmalonate (4mM)				
	Phosphate omitted				11.5
α-Oxoglutarate (5mM)	None				
	DL-*threo*-β-Hydroxyaspartate (4mM)	2.0	2.3	—	1.1
					1.7

Table 6. *Final extent of uptake at equilibrium of tricarboxylate-cycle anions by different types of yeast mitochondria*

The equilibrium extent of anion uptake at an external substrate concentration of 1mM was determined in the presence and in the absence of phosphate (2mM), as described in the Experimental section. The extent of uptake is expressed (a) in nmol·mg of protein^{-1} and (b) as the intramitochondrial concentration (mM), assuming that the anions are entirely accumulated within the sucrose-impermeable space whose volume (μl·mg of mitochondrial protein^{-1}) was determined in independent experiments as described in the Experimental section.

Mitochondria	Phosphate	Extent of uptake of substrates: L-Malate (a)	L-Malate (b)	Succinate (a)	Succinate (b)	Citrate (a)	Citrate (b)	α-Oxoglutarate (a)	α-Oxoglutarate (b)	Sucrose-impermeable space
Type 1	+	3.4	1.21	7.6	2.71	16	5.7	4.4	1.57	2.8
	−	2.0	1.25	4.2	2.63	14	8.8	3.9	2.44	1.6
Type 2	+	3.1	1.11	4.6	1.65	14	5.0	2.7	0.97	2.4
	−	2.0	1.25	3.0	1.88	13	8.1	2.4	1.50	1.3
Type 3	+	3.0	1.07	6.6	2.36	21	7.5	6.0	2.15	2.7
	−	1.8	1.12	4.4	2.76	19	11.9	5.5	3.44	1.7
Type 4	+	2.7	0.97	6.2	2.22	26	9.3	4.8	1.72	2.6
	−	2.2	1.37	3.9	2.44	24	15	3.9	2.45	1.8
Type 5	+	3.6	1.29	6.2	2.22	14	5.0	5.5	1.97	2.5
	−	2.0	1.25	3.7	2.31	12	7.5	5.1	3.20	1.6

properties identical with those of Type 3 mitochondria. Both Type 3 and Type 4 mitochondria are catabolite-repressed owing to growth on glucose medium, but have very similar anion-transport systems to those of de-repressed Type 1 mitochondria. However, there are small increases in the K_m values for L-malate, citrate and α-oxoglutarate uptake compared with those for Type 1 mitochondria. All four transport systems respond to inhibitors and activators of anion uptake in the same way as Type 1 mitochondria.

Table 5 shows that petite Type 5 mitochondria possess functional carrier systems for the four tricarboxylate-cycle anions. The kinetic parameters of

the four systems closely resemble those of other catabolite-repressed mitochondria from glucose-grown cells (Types 3 and 4), and the only minor differences are moderate decreases in the K_m and $V_{80,max.}$ values for the uptake of α-oxoglutarate.

Extent of accumulation of tricarboxylate-cycle anions

Table 6 summarizes the maximal extents of uptake at equilibrium of L-malate, succinate, citrate and α-oxoglutarate by the five types of yeast mitochondria. The extents of uptake of all four substrates are similar in Types 1, 3, 4 and 5 mitochondria, but somewhat lower for succinate and α-oxoglutarate in Type 2 mitochondria. In the absence of phosphate the extents of uptake of L-malate and succinate are appreciably lower and the uptakes of citrate and α-oxoglutarate are slightly decreased in all five types of mitochondria. However, the sucrose-impermeable space of the mitochondria increases in the presence of phosphate. By making the assumption that the tricarboxylate-cycle anions are accumulated entirely within the sucrose-impermeable space of the mitochondrial matrix the intramitochondrial concentrations of the substrates were calculated and are given in columns (b) of Table 6. The apparent stimulation of the extents of uptake of L-malate and succinate are due to the expansion of the mitochondrial matrix, as phosphate does not increase the intramitochondrial concentration of the substrate anions. With citrate and α-oxoglutarate, the presence of phosphate actually decreases the intramitochondrial concentration of the substrate anions. The calculated ratios of intramitochondrial to extramitochondrial concentrations of anion at equilibrium are greater than 1.0 for all four substrates. Further, the extent of accumulation of the substrates is comparable in Types 1, 3, 4 and 5 mitochondria, although it is somewhat lower in Type 2 mitochondria. The ratio of internal to external concentration are in the range 1.0–1.4 for L-malate, 1.7–2.8 for succinate, 1.0–3.4 for α-oxoglutarate and 5.0–15 for citrate.

Discussion

Validity of the determination of empirical kinetic parameters for anion uptake by yeast mitochondria

It is emphasized that the K_m and $V_{80,max.}$ values determined from v_{80} rates of substrate-anion uptake are purely empirical, and mainly intended to allow comparison between the carrier systems of different types of yeast mitochondria. Since the kinetics are determined at the approach to equilibrium for anion uptake, v_{80} values are much less than true initial rates of uptake. Moreover, the apparent K_m values determined by this method are greater, for example, than the K_m values obtained from initial uptake rate determinations in mammalian mitochondria (Haslam & Krebs, 1968), because the K_m values for extent of uptake at equilibrium are very high and increase the apparent K_m values in the approach to equilibrium situation. Nevertheless, the empirical K_m values obtained in the present work are not distorted too much, as they are considerably smaller than the equilibrium K_m values, and represent a valid approximation of the comparative kinetic properties of the carrier systems in different types of yeast mitochondria.

Properties of the tricarboxylate-cycle-anion-carrier systems of fully functional yeast mitochondria

The factors affecting the rate of uptake of L-malate, succinate, citrate and α-oxoglutarate by fully functional (Type 1) yeast mitochondria are very similar to those that control anion transport by mammalian mitochondria. Carrier-mediated transport of L-malate, succinate, citrate and α-oxoglutarate is evidenced by saturation kinetics of the concentration-dependence, and by the competitive inhibition caused by characteristic substrate analogues. In mammalian mitochondria the uptake of L-malate and succinate is mediated by a common carrier, which is stimulated by phosphate and inhibited by a number of substrate analogues including 2-*n*-butylmalonate, 2-benzylmalonate and 2-methylsuccinate (Chappell & Crofts, 1966; Chappell & Robinson, 1968). Our present results indicate that in yeast mitochondria the entry of L-malate and succinate is mediated by a common carrier as evidenced by (1) the mutual competition for uptake by L-malate and succinate, (2) the comparable stimulation of rates of uptake of L-malate and succinate by phosphate, (3) the comparable inhibitions by 2-*n*-butylmalonate and 2-methylsuccinate of rates of uptake of both L-malate and succinate. On the other hand, Kolarov *et al.* (1972) have proposed that yeast mitochondria have different carriers for L-malate and succinate. However, the latter authors did not attempt to measure the kinetic parameters of the carrier systems for L-malate and succinate, which are reported in the present studies.

The presence of a citrate carrier similar in properties to that of mammalian mitochondria is evidenced by competition with isocitrate, the requirement for both phosphate and L-malate for optimum uptake of citrate, and by the abolition of L-malate stimulation by 2-*n*-butylmalonate. A distinct carrier for α-oxoglutarate is suggested by the specific inhibition by DL-*threo*-β-hydroxyaspartate and glutarate. However, the α-oxoglutarate carrier of yeast mitochondria differs from the corresponding mammalian system in two respects: (1) it is moderately stimulated by phosphate, which has no direct effect on the mammalian system; (2) it is not affected by aspartate, which inhibits the mammalian system (Chappell, 1968; Chappell & Robinson, 1968; Robinson & Chappell,

1967). The α-oxoglutarate carrier is distinct from the dicarboxylate and citrate carriers, as it is inhibited by DL-*threo*-β-hydroxyaspartate, which has no effect on the other two systems.

The maximal extents of uptake of L-malate, succinate and α-oxoglutarate by Type 1 mitochondria are very similar to those obtained by van Dam & Tsou (1968) for rat liver mitochondria, and the amount of anionic charge is approximately equivalent to the positive charge of intramitochondrial K^+, whose concentration is approx. 70mM (J. M. Haslam, unpublished work). The maximum extent of citrate uptake (>90mM) is considerably greater than that of the other anions, and higher than that obtained with mammalian mitochondria (van Dam & Tsou, 1968). The reasons for this have not been established, but it is unlikely that the large amounts of citrate are present in the sucrose-impermeable space alone, as the matrix does not contain sufficient cations to neutralize the negative charges of the citrate. The inhibition of both the rate and the extent of L-malate uptake by 2-methylsuccinate and 2-*n*-butylmalonate suggests that these anions not only compete for the carrier to inhibit the rate of uptake, but are themselves accumulated and inhibit the extent of uptake of L-malate by competing for exchange with endogenous anions in the mitochondrial matrix.

Yeast mitochondria are capable of accumulating the four tricarboxylate-cycle anions against their concentration gradients. Since respiration is inhibited by antimycin A, the only energy available to support the accumulation of the anions is from the pre-existing gradients of endogenous anions in the mitochondria. Rat liver mitochondria have a similar capacity to accumulate substrates in the matrix space under conditions where the energy cannot be generated by oxidative phosphorylation (van Dam & Tsou, 1968). The uptake of substrate anions comprises both binding to specific sites and exchange with endogenous anions. The latter probably predominates, but our methods do not distinguish between the two processes. The increase in the extents of uptake of L-malate and succinate caused by phosphate is due to the expansion of the sucrose-impermeable space, as the calculated intramitochondrial concentration of the substrate anions is virtually unaffected by phosphate. However, the concentrations of α-oxoglutarate and citrate accumulated by mitochondria are diminished in the presence of phosphate, probably owing to competition of the phosphate anion for the exchangeable anion of the mitochondrial matrix.

Effects of genetic and physiological manipulation on the uptake of the tricarboxylate-cycle anions

Despite the gross changes in the mitochondrial membrane composition produced by anaerobiosis (Type 2), catabolite repression (Type 3), inhibition of mitochondrial protein synthesis (Type 4) and the elimination of mitochondrial DNA (Type 5), the transport systems for L-malate, succinate, citrate and α-oxoglutarate are present in all four modified types of yeast mitochondria and exhibit essentially similar properties to those of fully functional (Type 1) mitochondria. There are small variations in the kinetic and equilibrium parameters for anion uptake in certain instances. The largest changes occur in the carrier system for α-oxoglutarate, whose K_m value is appreciably lower in Type 5 mitochondria (2mM), and higher (10mM) in Type 3 mitochondria, than in Type 1 mitochondria (4mM), but these differences are within the range of experimental variation and are probably not significant. The parameters for L-malate uptake also show a small variation, but those for succinate and citrate are virtually indistinguishable in the five types of mitochondria. These minor differences in the properties of the carrier systems probably reflect some reorganization of the inner mitochondrial membrane in which the carrier systems are situated, owing to the gross changes in composition of the different mitochondrial types.

The extents of uptake of the four substrate anions by the different types of mitochondria are markedly similar. Only in anaerobic (Type 2) mitochondria is the accumulation of succinate, citrate and α-oxoglutarate somewhat less than that of the other mitochondrial types. Further, all five types of mitochondria are capable of accumulating the tricarboxylate anions against their concentration gradients to a comparable degree, suggesting that the different mitochondrial types contain similar amounts of exchangeable anion.

Genetic origin and site of synthesis of the tricarboxylate-cycle-anion-transport systems

Since petite (Type 5) mitochondria lack mitochondrial DNA but possess active and apparently normal carrier systems for L-malate, succinate, citrate and α-oxoglutarate, it is concluded that all the protein components of the transport systems are coded by nuclear genes. Moreover, inhibition of the mitochondrial protein-synthesizing system by erythromycin in Type 4 mitochondria, or the total absence of mitochondrial ribosomes in Type 5 mitochondria caused by the loss of rRNA genes, does not alter the anion-transport systems. This shows that all the proteins of the transport systems are synthesized extramitochondrially by cell-sap ribosomes. The presence of normal anion-transport systems in anaerobic (Type 3) mitochondria and in petite (Type 5) mitochondria, together with the knowledge that both these types of mitochondria possess tricarboxylate-cycle enzymes (for review see Linnane & Haslam,

1970), probably reflects that even respiratory-deficient mitochondria have an important function in intermediary metabolism, for example in the synthesis of amino acids.

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