A Comparative Study of Cells and Mitochondria of *Saccharomyces cerevisiae* and of a Hydrocarbon-utilizing Yeast, *Candida lipolytica*

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SUMMARY

The properties of cells and mitochondria isolated from *Candida lipolytica*, a hydrocarbon-utilizing yeast, grown on ethanol, glucose and *n*-alkanes were examined. A comparative study was made of the properties of the whole cells and mitochondria of *C. lipolytica* and of those of *Saccharomyces cerevisiae*.

The reduced-minus-oxidized cytochrome spectrum of *C. lipolytica* grown on ethanol showed a much larger amount of cytochromes *aa*₃ and a very broad cytochrome *b*-type absorption band compared with *S. cerevisiae*. The relative amounts of cytochromes in *C. lipolytica* on hydrocarbons differed according to the growth phase but never reached the levels observed in the cells grown on ethanol. Furthermore, as judged by the reduced-minus-oxidized cytochrome spectra, *C. lipolytica* was much less affected by glucose repression than was *S. cerevisiae*. The fatty acids of mitochondria isolated from ethanol-grown *C. lipolytica* consisted mainly of equal amounts of oleic (C_{18:1}) and linoleic (C_{18:2}) unsaturated fatty acids, each making up about 40 % of the total. In contrast, *S. cerevisiae* mitochondria contained palmitoleic (C_{16:1}) (approx. 45 %) and oleic (approx. 35 %) as the main fatty-acid components. There was an increase in the amount of oleic acid (60 %) relative to linoleic acid (20 %) when *C. lipolytica* was grown on even-numbered hydrocarbons and a progressive increase in the amount of heptadecenoic acid (C_{17:1}) up to 75 % when grown on odd-numbered *n*-alkanes of increasing carbon-chain length from C₁₁ to C₁₅. The changes in fatty-acid composition were correlated with changes in membrane fluidity as measured by differences in transition temperatures in Arrhenius plots of mitochondrial membrane-bound enzymes.

The ATPases of *C. lipolytica* and *S. cerevisiae* mitochondria were examined, and marked differences in specific activity (3- to 5-fold higher in *S. cerevisiae*), pH profile and oligomycin sensitivity were noted.

INTRODUCTION

There has recently been much interest in micro-organisms, including yeasts, capable of utilizing hydrocarbons as sole carbon source. There are now several commercial operations for the production of yeast for the preparation of animal feedstuffs from waste paraffins obtained in the fractionation of crude oil (Wilkinson, 1971). The degradation of aliphatic hydrocarbons by micro-organisms has been discussed by Klug & Markovetz (1970) and the breakdown of *n*-alkanes by yeast cells has recently been described (Gallo, Roche, Aubert & Azoulay, 1973*a*; Gallo, Bertrand, Roche & Azoulay, 1973*b*). The hydrocarbon is thought to proceed to the primary alcohol and then through the aldehyde to the fatty acid. It is presumed that it is then broken down in the mitochondria by β -oxidation.

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There have been few reports on the properties of mitochondria from hydrocarbon-utilizing yeast. The present studies were undertaken to describe some of the properties of intact cells and mitochondria from *C. lipolytica* grown on different substrates which included *n*-alkanes of chain length C_{10} to C_{16} . The properties of the cells and mitochondria of *C. lipolytica* were compared with those of *S. cerevisiae*. Marked differences in cytochrome content, fatty-acid composition and in the activities of mitochondrial membrane-bound enzymes were observed. Preliminary accounts of parts of this work have been reported (Skipton, Watson, Houghton & Griffiths, 1973; Houghton, Skipton, Watson & Griffiths, 1973).

METHODS

Growth of organisms. *Candida lipolytica*, strain YB423 (B.P. Chemicals, Sunbury-on-Thames, Middlesex), was grown in a fermenter (New Brunswick Scientific Co., New Brunswick, New Jersey, U.S.A.) at 30 °C in a medium containing (per litre): 0.5 % (w/v) yeast extract; 2.5 g NH_4Cl ; 7.0 g KH_2PO_4 ; 3.0 g $Na_2HPO_4 \cdot 2H_2O$; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.1 g $NaCl$; 0.5 mg HBO_3 ; 0.4 mg $CuSO_4 \cdot 5H_2O$; 0.1 mg KI ; 0.4 mg $MnSO_4$; 0.2 mg $Na_2MoO_4 \cdot 2H_2O$; 0.4 mg $ZnSO_4 \cdot 7H_2O$; 0.2 mg $FeCl_3$. $CaCl_2 \cdot 2H_2O$ (0.1 g) was added after autoclaving. Ethanol (0.5 %, v/v), glucose (5.0 %, w/v) or *n*-alkane (0.1 to 0.4 %, v/v) was used as carbon source as indicated in the text. Tributyl citrate at 0.1 to 0.2 ml/l was used as antifoaming agent, and the pH of the medium was between 5.8 and 6.0. Stirring speed was 500 rev./min and compressed air was passed through the culture at 1 l air/min/l medium. *Saccharomyces cerevisiae*, strain D22, was grown under the same conditions except that the mineral salts were according to Wickerham (1946) and adenine sulphate (0.1 g/l) was added to the medium. A 1.0 % inoculum from a starter culture grown for 24 h on 0.5 % (v/v) ethanol was used.

The yeasts were also grown on ethanol (0.5 %, v/v) or glucose (5.0 %, w/v) in 400 to 500 ml medium in 2 l conical flasks at 30 °C and shaken at 250 rev./min on a Mark 5 shaker (L.H. Engineering Co., Stoke Poges, Buckinghamshire). These cultures were normally used for cytochrome determinations as indicated in the text.

Isolation of mitochondria. Yeasts were harvested at exponential or early stationary phase as indicated in the text, washed, and then resuspended in buffer containing 0.5 M-sorbitol, 20 mM-tris-HCl, 1 mM-EDTA and bovine serum albumin (BSA) (0.2 %, w/v) at pH 8.0. They were broken with glass beads (0.45 to 0.5 mm diam) for 15 s in a Braun shaker (B. Braun Apparatebau, Melsungen, Germany) at 4000 rev./min. Mitochondria were isolated from the homogenate by differential centrifugation and further purified on a discontinuous 20 to 70 % (w/v) sucrose gradient containing 20 mM-tris-HCl, 1 mM-EDTA pH 7.5. Gradients were run at 24000 rev./min for 2.5 h in the SW 25.1 rotor of a Beckman Model L2 ultracentrifuge. The main mitochondrial band was collected and resuspended in buffer containing 0.25 M-sorbitol, 20 mM-tris-HCl, 1 mM-EDTA pH 7.5 at a protein concentration of approximately 15 mg/ml. Mitochondria were also prepared by digestion of the yeast wall with snail-gut enzymes and disruption of the protoplasts in a French pressure cell, as previously described (Watson, Haslam & Linnane, 1970).

Estimation of fatty acids. Harvested, washed yeast (1.0 to 2.0 g wet wt) or 20 to 40 mg mitochondria were resuspended in 3 to 5 ml of 40 % (w/v) potassium hydroxide and refluxed for 4 h to saponify the lipids. Fatty-acid esters were prepared and determined by gas-liquid chromatography on a polyethyleneglycol succinate column as previously described (Watson, Bertoli & Griffiths, 1973a). Relative percentages of fatty acids were

determined by measurement of the peak areas, estimated from peak height $\times$ one-half peak base.

Cytochrome spectra. The spectra were recorded on a Unicam SP1800 spectrophotometer in 1 cm light path cells (final volume 2.5 to 3.0 ml) using the turbid solution cell position and a slit width of 0.6 mm. The sample cell was reduced with a few grains of dithionite and the reference cell was oxidized with 10 μ l of 100 vol. H_2O_2 . Difference spectra of mitochondria were recorded under the same conditions.

Succinate oxidase and NADH oxidase assays. Oxygen uptake was measured by means of a thermostatted oxygen electrode. Two ml of air-equilibrated buffer containing 50 mM-potassium phosphate pH 7.4 was used for each assay; 50 to 200 μ l of mitochondrial suspension (0.5 to 1.5 mg protein) was added and, after a further minute, 0.4 mM-ADP followed by either NADH (2.5 mM) or succinate (20 mM) as substrate.

Succinate dehydrogenase assay. A Cary 14 spectrophotometer fitted with a Churchill refrigerated thermostat was used to assay the enzyme. Accurate temperature measurement was by means of a thermocouple ($\pm 0.1^\circ C$) which fitted directly into the cuvette (1.0 cm light path). The reaction mixture contained, in a final volume of 1.1 ml, 50 mM-potassium phosphate, 50 to 100 μ g mitochondrial protein, 25 mM-succinate and 2 mM-KCN, pH 7.4. Incubation was for 5 min and then the reaction was started by the rapid addition of 0.05 mM-2,6-dichlorophenol-indophenol (DCPIP) and 0.5 mM-phenazine methosulphate (PMS). The decrease in absorbance of DCPIP at 600 nm was followed. An extinction coefficient of $\epsilon_{\text{nm}} = 21$ was used (King & Howard, 1967). A current of dry nitrogen was passed through the cell chamber to prevent condensation at the lower temperatures.

Mitochondrial ATPase assay. The ATPase was assayed at $30^\circ C$ as previously described (Houghton *et al.* 1973).

Dry weight and protein estimations. Dry weights of intact yeasts were measured after filtration on to Whatman GF/C glass-fibre paper (2.4 cm diam) and drying in an oven at 110 to $120^\circ C$ for 24 h. Protein estimations were by the procedure of Lowry, Rosebrough, Farr & Randall (1951).

Materials. ADP, ATP, PMS, DCPIP, NADH and oligomycin were obtained from Sigma. Methyl ester standards of fatty acids were obtained from Sigma or from BDH. All other chemicals were of analytical reagent grade where available.

RESULTS

As shown in Fig. 1 the growth curves of *Candida lipolytica* and *Saccharomyces cerevisiae* on ethanol were essentially the same. The lag phase of about 10 h may be contrasted with the much longer lag phase of about 18 h when *C. lipolytica* was grown on tetradecane.

Candida lipolytica can utilize hydrocarbon as sole carbon source and is able to grow in liquid culture on *n*-alkanes of 10 to 20 carbon atoms in chain length. Most of the studies on the growth of yeast on hydrocarbons, in order to determine substrate specificity, have been done using *Candida* species. Differences in cell yield on *n*-alkanes of varying chain length do not follow any general rule (Klug & Markovetz, 1970). In the measurements of growth of *C. lipolytica* on several *n*-alkanes in batch culture (Fig. 2) it was noticeable that there was no large increase in cell yield in going from *n*-decane to *n*-tetradecane and *n*-hexadecane. Furthermore, when the concentration of *n*-tetradecane was increased fourfold there was less than a twofold increase in the cell yield (Fig. 2). These results may be contrasted with those for cells grown on ethanol where there was an approximate doubling in yield in going from 0.5 to 1.0 % (v/v) ethanol (results not shown).

Fig. 1. Growth curves of *S. cerevisiae* and *C. lipolytica* in fermenters. Δ , *S. cerevisiae* and, $\blacktriangle$, *C. lipolytica* grown on ethanol (0.5 %, v/v). $\square$, *C. lipolytica* grown on *n*-tetradecane (0.1 %, v/v).

Fig. 2. Growth curves of *C. lipolytica* on various *n*-alkanes. $\blacksquare$, Decane (0.1 %, v/v); $\square$, tetradecane (0.1 %, v/v); $\circ$, hexadecane (0.1 %, v/v); $\bullet$, tetradecane (0.4 %, v/v).

There are two basic ways in which uptake of hydrocarbons by yeast cells can occur. This may be either by direct contact of the cells with paraffin droplets or by transfer from the droplets to the cells through the aqueous phase. Evidence for the former mechanism has been presented by several authors (see Klug & Markovetz, 1970). On the other hand,

Table 1. *Fatty-acid composition of intact yeast cells grown on different carbon sources*

Substrate	< C ₁₅	C ₁₅	C ₁₆	C ₁₇	C ₁₈	C _{16:1}	C _{17:1}	C _{18:1}	C _{18:2}	Total unsaturated fatty acids (%)
<i>Candida lipolytica</i>										
Ethanol	—	—	7	1	1	6	6	38	41	91
Glucose (exponential phase)	3	—	11	—	2	5	2	39	38	84
Decane	—	—	8	—	—	3	2	64	23	92
Dodecane	—	—	9	—	2	9	3	61	15	88
Tetradecane	—	—	7	—	—	9	4	57	18	87
Hexadecane	—	—	7	—	—	9	1	68	14	92
Undecane	—	—	9	—	—	8	11	60	10	89
Tridecane	—	7	3	2	1	5	32	39	7	83
Pentadecane	—	5	1	3	—	—	72	12	7	91
<i>Saccharomyces cerevisiae</i>										
Ethanol	—	—	11	—	4	47	—	36	—	83
Glucose (exponential phase)	3	—	13	—	5	44	—	36	—	80

Fatty acids are shown as percentages of total fatty acids and denoted by the convention, number of carbon atoms:number of double bonds. All cells were harvested at stationary phase unless otherwise shown.

Goma, Pareilleux & Durand (1973) have recently shown that the most soluble *n*-alkane, present either as the sole carbon source or in a mixture of 2 or 8 *n*-alkanes, was always the first to be utilized. In the present studies, it was observed that *n*-decane was taken up much more rapidly than *n*-tetradecane or *n*-hexadecane (Fig. 2). These results could be interpreted as indicating that the hydrocarbons were utilized primarily in the dissolved state, since the solubility of *n*-decane in aqueous solution is much greater than that of the other hydrocarbon substrates (McAuliffe, 1969). However, when the concentration of the hydrocarbon substrate was increased, the lag phase of growth was also prolonged (Fig. 2). It is clear, therefore, that other factors such as the physical state of the hydrocarbon droplets have to be taken into consideration in proposing a mechanism for hydrocarbon uptake by yeast (Moo-Young, Shimizu & Whitworth, 1971). It is possible that hydrocarbon utilization may proceed by both mechanisms, i.e. in the dissolved state and also by direct contact, the exact mechanism depending on such parameters as physical state of the hydrocarbon substrate, phase of growth, temperature, etc.

Cell fatty acids

Under all conditions of growth on ethanol, glucose or *n*-alkane as substrate the *C. lipolytica* cells contained between 80 and 90 % of the total fatty acid as unsaturated fatty acid (Table 1). The major components were oleic (C_{18:1}) and linoleic (C_{18:2}) unsaturated fatty acids when *C. lipolytica* was grown on ethanol, glucose or even-numbered *n*-alkanes. There were, however, significant differences in the relative amounts of oleic and linoleic fatty acids depending on the nature of the substrate. When *C. lipolytica* was grown on ethanol or glucose, the ratio of oleic to linoleic was about 1:1 and these made up approximately 80 % of the total fatty acids, with small amounts of palmitic and palmitoleic acids and traces of heptadecenoic and stearic acids accounting for the rest. In *Candida* grown on even-numbered *n*-alkanes from decane to hexadecane the ratio of oleic to linoleic was in all cases about 3:1. These were again the major fatty-acid constituents (80 % of the total) with small amounts of palmitic, palmitoleic, heptadecenoic and stearic acids account-

Table 2. Fatty-acid composition of mitochondria isolated from *Saccharomyces cerevisiae* and *Candida lipolytica*

Substrate	< C ₁₅	C ₁₅	C ₁₆	C ₁₇	C ₁₈	C _{16:1}	C _{17:1}	C _{18:1}	C _{18:2}	Total unsaturated fatty acids (%)
<i>S. cerevisiae</i>										
Ethanol	—	—	13	—	7	45	—	35	—	80
<i>C. lipolytica</i>										
Ethanol	2	—	9	1	1	9	1	40	37	87
Tetradecane	1	—	9	—	2	7	3	55	22	87
Pentadecane	3	6	4	—	—	3	56	19	9	87

Fatty acids are shown as percentages of total fatty acids and denoted by the convention, number of carbon atoms: number of double bonds.

ing for the rest. There were no significant changes in the cell fatty-acid profiles of *C. lipolytica* at different growth phases in batch culture on ethanol or hydrocarbons under our conditions of growth. *Candida lipolytica* grown on odd-numbered *n*-alkanes showed an increasing incorporation (from 11 % of the total to 72 %) of heptadecenoic acid (C_{17:1}) in going from undecane to tridecane to pentadecane, with a corresponding decrease in the amounts of oleic and linoleic acids (Table 1). It was possible to demonstrate a linear relationship between the relative amount of fatty acids with eighteen carbon atoms after, growth on odd-numbered *n*-alkanes, and the number of carbon atoms in the hydrocarbon chain, i.e. in going from C₁₁ to C₁₃ to C₁₅, the C₁₈ fatty acids made up 70, 47 and 19 %, respectively, of the total fatty acids. Hug & Fiechter (1973) have also shown a similar relationship in *Candida tropicalis* grown on *n*-alkanes. The biosynthesis of C₁₈ fatty acids, at least in the case of *Candida* sp. grown on odd-numbered *n*-alkanes, therefore appears to be dependent on the chain length of the *n*-alkane substrate.

Table 1 summarizes the fatty-acid composition of *C. lipolytica* grown on different substrates. For comparison we have included the fatty-acid profile of *S. cerevisiae* grown on ethanol and on glucose. In contrast to *C. lipolytica* cells, those of *S. cerevisiae* were extremely rich in palmitoleic acid (C_{16:1}), which accounted for about 45 % of the total cell fatty acid, with oleic acid (C_{18:1}) accounting for about 35 %.

Mitochondrial fatty acids

The fatty-acid composition of *C. lipolytica* mitochondria (Table 2) was similar to that of the corresponding intact cells. Large amounts of heptadecenoic acid (C_{17:1}) were present in the mitochondrial membranes of pentadecane-grown *C. lipolytica* cells.

The marked differences in the fatty-acid composition of *S. cerevisiae* and *C. lipolytica* mitochondria raised the possibility of differences in biochemical properties of the mitochondria from the two species of yeast. While the biochemical properties of *S. cerevisiae* mitochondria are well documented (Ohnishi *et al.* 1967; Kovac, Bednarova & Greksak, 1968; Mackler & Haynes, 1973) there have been few reports on *C. lipolytica* mitochondria, particularly from cells grown on hydrocarbons.

Fig. 3. Arrhenius plots of membrane-bound respiratory enzymes of mitochondria isolated from cells grown on ethanol. The specific activities were expressed as nmol substrate/min/mg protein for NADH oxidase and succinate oxidase, and as nmol DCPIP/min/mg protein for succinate dehydrogenase. *Candida lipolytica*: $\circ$, NADH oxidase; $\triangle$, succinate oxidase; $\square$, succinate dehydrogenase. *Saccharomyces cerevisiae*: $\bullet$, NADH oxidase; $\blacktriangle$, succinate oxidase; $\blacksquare$, succinate dehydrogenase.

Arrhenius plots

The effect of temperature on the activities of *C. lipolytica* and *S. cerevisiae* mitochondrial membrane-bound enzymes was examined. These studies were prompted by the differences in fatty-acid composition in the two mitochondrial species and by previous observations which showed that phase-transition temperatures in *S. cerevisiae* mitochondria are influenced by the fatty-acid composition of the membrane lipids (Ainsworth, Tustanoff & Ball, 1972; Watson *et al.* 1973*a*).

Figure 3 shows Arrhenius plots of the logarithms of specific activities plotted against the reciprocal of the absolute temperature for NADH and succinate oxidases of mitochondria isolated from ethanol-grown cells. In *C. lipolytica* mitochondria the phase-transition temperatures at which there was an increase in activation energy were in the range 14 to 17 $^{\circ}\text{C}$. By comparison the corresponding mitochondrial membrane-bound enzymes of *S. cerevisiae* showed transition temperatures in the range 17 to 20 $^{\circ}\text{C}$. These differences in transition temperatures were observed over 4 or 5 separate mitochondrial preparations for both yeast

Table 3. *Transition temperatures and activation energies of Candida lipolytica and Saccharomyces cerevisiae mitochondrial membrane-bound enzymes*

		Specific activity (nmol substrate/ min/mg)	Transition temperature (°C)	Activation energy (kcal/mol)	
Enzyme				Above transition temperature	Below transition temperature
<i>C. lipolytica</i>					
Ethanol	NADH oxidase	2140	14-17	2.2	8.4
	Succinate oxidase	980	14-17	8.2	16.5
	Succinate dehydrogenase	4000	14-17	21.2	36.9
Tetradecane	NADH oxidase	520	17-20	3.5	10.1
	Succinate oxidase	230	17-20	10.4	15.2
	Succinate dehydrogenase	1300	15-18	30.2	38.3
<i>S. cerevisiae</i>					
Ethanol	NADH oxidase	800	17-20	6.2	20.4
	Succinate oxidase	410	17-20	14.7	23.9
	Succinate dehydrogenase	1100	17-20	20.1	37.8

species. In general there was a two- to threefold increase in activation energy below the transition temperature (Table 3). Also, the specific activities of the NADH and succinate oxidases in *C. lipolytica* mitochondria were several-fold higher than those of the *S. cerevisiae* mitochondria (Table 3). A general characteristic of mitochondria from both yeast species was the lower activation energy, especially above the transition temperature, of the NADH oxidase as compared with the succinate oxidase system. As a result the NADH oxidase was still relatively active at temperatures well below the transition temperature (Fig. 3). By comparison, the succinate oxidase activity decreased rapidly at temperatures below the transition point (Fig. 3). A preliminary account of the lower activation energy of the NADH oxidase system has been presented (Watson, Bertoli & Griffiths, 1973*b*).

Succinate dehydrogenase showed a similar non-intersecting discontinuity in Arrhenius plots (Fig. 3). Here again the specific activity of the *C. lipolytica* mitochondria was several-fold higher than the *S. cerevisiae* mitochondria. The activation energies, both above and below the transition temperatures, for both types of mitochondria, were similar (Table 3).

Arrhenius plots of the corresponding respiratory enzymes in mitochondria isolated from *C. lipolytica* grown on tetradecane are shown in Fig. 4. All three mitochondrial membrane-bound enzymes had lower specific activities and higher transition temperatures than those from mitochondria isolated from *C. lipolytica* grown on ethanol. The mitochondria from the tetradecane-grown cells were comparable in terms of specific activity to those of *S. cerevisiae* mitochondria. The transition temperatures were also in the same range (Table 3). The activation energies of the mitochondrial enzymes, above and below the transition temperatures, increased in the order NADH oxidase, succinate oxidase, succinate dehydrogenase, as they do in mitochondria isolated from ethanol-grown cells (Table 3). The activation energy of the succinate dehydrogenase of mitochondria isolated from tetradecane-grown cells was extremely high. However, it was not clear whether this observation was due to an artefact of the isolation procedure, since mitochondria from tetradecane-grown cells were prepared only by the mechanical-breakage procedure.

Fig. 4. Arrhenius plots of membrane-bound respiratory enzymes of mitochondria isolated from *C. lipolytica* cells grown on tetradecane. Specific activities were expressed as nmol/min/mg protein. Δ , NADH oxidase; $\blacktriangle$, succinate oxidase; $\bullet$, succinate dehydrogenase.

Cytochrome spectra

The reduced-minus-oxidized cytochrome spectra of *C. lipolytica* and *S. cerevisiae* cells grown on ethanol in shake culture are shown in Fig. 5. The *C. lipolytica* cells were characterized by a large cytochrome aa_3 absorption peak at 605 nm and a relatively broad cytochrome b -type absorption in the 560 to 565 nm region. The differences in the cytochrome pattern of *C. lipolytica* compared with *S. cerevisiae* were further emphasized when the Soret bands were examined. In *Candida* cells there was a large absorption maximum at 445 nm characteristic of cytochromes aa_3 , and a small shoulder in the 415 to 430 nm region due to reduced cytochrome c (415 nm) and cytochrome b (425 nm). *Saccharomyces* cells in contrast showed a peak in the Soret region at 445 nm characteristic of cytochromes aa_3 and a somewhat larger and broader absorption in the region 415 to 430 nm, indicating a large amount of cytochrome c relative to cytochromes b and aa_3 .

The cytochrome spectra of *C. lipolytica* grown on high glucose concentrations were examined, since under this condition *S. cerevisiae* shows a highly repressed cytochrome

Fig. 5. Cytochrome reduced-minus-oxidized spectra of *C. lipolytica* and *S. cerevisiae* grown in shake culture on ethanol (0.5 %, v/v) to early stationary phase, and of *C. lipolytica* grown on *n*-tetradecane (0.2 %, v/v) fermenter. I, *C. lipolytica* on ethanol (25 mg dry wt/ml); II, *S. cerevisiae* on ethanol (31 mg dry wt/ml); III, *C. lipolytica* on *n*-tetradecane (stationary phase, 28.5 mg dry wt/ml); IV, *C. lipolytica* on *n*-tetradecane (log phase, 205 mg dry wt/ml).

spectrum (Jayaraman, Cotman, Mahler & Sharp, 1966). The cytochrome spectra of *Candida* and *Saccharomyces* cells grown on 5 % (w/v) glucose and harvested in the exponential phase are shown in Fig. 6. *Saccharomyces cerevisiae* showed a glucose-repressed cytochrome spectrum while *C. lipolytica* showed a spectrum more typical of derepressed cells (cf. Fig. 5). Although, on a cell dry weight basis, there were less cytochromes in the glucose-grown as compared with the ethanol-grown *Candida* cells, the *C. lipolytica* cells were obviously less subject to glucose repression than *S. cerevisiae*. Indeed the cytochrome content (again on a cell dry weight basis) of *C. lipolytica* grown on glucose compared favourably with that of fully derepressed *S. cerevisiae* grown on ethanol (cf. Table 4). The cytochrome aa_3 content of glucose- and ethanol-grown *C. lipolytica* cells was about five- and tenfold respectively higher than that of *S. cerevisiae* grown on ethanol.

Quite different cytochrome patterns were observed when *C. lipolytica* cells were grown on tetradecane. Figure 5 shows the reduced-minus-oxidized cytochrome spectra of tetradecane-grown cells harvested in exponential and stationary phases. The cytochrome spectra were

Fig. 6. Cytochrome reduced-minus-oxidized difference spectra of cells grown on glucose (5.0 %, w/v) in shake culture and harvested in exponential phase. Spectra were recorded as outlined in Methods. —, *C. lipolytica* (9 mg dry wt/ml); ----, *S. cerevisiae* (26 mg dry wt/ml).

typical of the change in going from a repressed (exponential phase) to a derepressed (stationary phase) state characteristic of glucose derepression in *S. cerevisiae* (Slonimski, 1953; Jayaraman *et al.* 1966). The changes in the cytochrome spectra in the Soret region were particularly dramatic. In the case of the exponential phase cells, the cytochromes aa_3 absorption peak (445 nm) appeared as a small shoulder on the large cytochrome b peak at 424 nm while in stationary phase the cytochromes aa_3 maximum was almost equal to the cytochrome b absorption.

Mitochondria

It was not possible, from small-scale shake cultures, to obtain sufficient mitochondria for all the biochemical studies. The majority of experiments, therefore, were conducted on mitochondria isolated from yeasts grown in large-scale fermenters (see Methods). The cytochrome content of fermenter-grown yeast was somewhat less than that of yeast grown in small-scale shake culture (cf. Table 4). However, the same general trends in cytochrome composition were observed.

Quantitative estimation of the cytochrome content of the isolated mitochondria was complicated by the variable loss of cytochrome c during the isolation procedures. This loss was apparent in mitochondria prepared both from mechanical disruption of cells and from protoplast lysates. Despite these difficulties it was still clear that mitochondria from *C. lipolytica* grown on ethanol had a much higher total cytochrome content than the corresponding mitochondria from *S. cerevisiae*. In particular the cytochromes b and aa_3 contents

Table 4. Cytochrome content of intact cells and mitochondria of *Candida lipolytica* and *Saccharomyces cerevisiae* grown on different substrates

	Cytochromes					
	<i>c</i>	<i>b</i>	<i>aa₃</i>	<i>c/c</i>	<i>b/c</i>	<i>aa₃/c</i>
Intact cells						
<i>C. lipolytica</i>						
Ethanol	0.230	0.124	0.168	1.00	0.54	0.73
Ethanol (fermenter)	0.180	0.083	0.089	1.00	0.46	0.49
Glucose (exponential phase)	0.180	0.074	0.084	1.00	0.41	0.46
Tetradecane						
(exponential phase, fermenter)	0.149	0.135	0.028	1.00	0.91	0.19
(stationary phase, fermenter)	0.159	0.097	0.059	1.00	0.61	0.37
<i>S. cerevisiae</i>						
Ethanol	0.160	0.026	0.017	1.00	0.17	0.11
Glucose (exponential phase)	0.040	0.026	0.0039	1.00	0.65	0.097
Mitochondria						
<i>C. lipolytica</i>						
Ethanol	1.36	0.840	0.740	1.00	0.62	0.54
Tetradecane	0.565	0.445	0.245	1.00	0.79	0.43
<i>S. cerevisiae</i>						
Ethanol	1.09	0.590	0.220	1.00	0.54	0.20

Cytochrome content of intact cells is shown as nmol/mg dry weight. Cytochrome content of mitochondria is shown as nmol/mg protein. The following extinction coefficients for cytochrome difference spectra were taken: cytochrome *c* 550–540 nm, $\epsilon_{\text{nm}} = 19$ (Wilson & Epel, 1968); cytochrome *b* 560–540 nm (Wilson & Epel, 1968), $\epsilon_{\text{nm}} = 22$; cytochrome *aa₃* 605–630 nm, $\epsilon_{\text{nm}} = 24$ (van Gelder, 1966).

Cells were grown in shake flasks (2 l) and harvested in stationary phase unless otherwise indicated, washed in distilled water and resuspended in 60% (w/v) sorbitol, 50 mM-phosphate, pH 7.0 (15–25 mg dry wt/ml).

Mitochondria were isolated from stationary-phase cells grown in fermenters, and suspended in 0.25 M-sorbitol, 20 mM-tris-HCl, 1 mM-EDTA, pH 7.5, to 4 mg protein/ml.

of *C. lipolytica* were about 1.5 and 3.5 times higher, respectively, than those of *S. cerevisiae*. The cytochrome levels in *S. cerevisiae* presented in Table 4 were comparable to those reported by Ohnishi *et al.* (1967) for *Saccharomyces carlsbergensis* mitochondria.

A comparison of the ratios of cytochromes in *S. cerevisiae* and *C. lipolytica* is summarized in Table 4. For cells grown on ethanol the ratio of cytochrome *c*:*b*:*aa₃* in *C. lipolytica* was 1.0:0.54:0.73 and in *S. cerevisiae* 1.0:0.17:0.11. These results clearly demonstrate that the *C. lipolytica* cells were comparatively rich in cytochromes *b* and *aa₃*, and the *S. cerevisiae* cells in cytochrome *c*. The corresponding ratios for cells grown on glucose indicated that while there was some repression of cytochromes *b* and *aa₃* in *C. lipolytica*, the *S. cerevisiae* cells were relatively rich in cytochrome *b* (cytochrome *c*:*b* ratio of 1.0:0.65); the cytochrome *c*:*aa₃* ratio of 1.0:0.10 was about the same as that of the ethanol-grown cells. In *S. cerevisiae* the amount of cytochrome *b*, at least on a dry weight basis, was the same in ethanol-grown and in glucose-repressed cells. On the other hand the amounts of cytochromes *c* and *aa₃* were about fourfold less in the case of the latter.

Candida lipolytica grown on tetradecane showed changes in the cytochrome ratios depending on the phase of growth. Stationary phase cells were relatively rich in cytochromes *aa₃* while exponential phase cells were extremely rich in cytochrome *b* (Table 4).

Cytochrome ratios in the isolated mitochondria were again complicated by some loss of cytochrome *c* during the isolation procedures. Nevertheless, the relatively high cytochrome *b*

Table 5. *Percentage inhibition of mitochondrial ATPase by oligomycin at various pH values*

		Oligomycin ($\mu\text{g}/\text{mg}$ protein)		
	pH	2	10	50
<i>Saccharomyces cerevisiae</i>				
Ethanol	7.0	11	38	88
	9.5	57	86	95
<i>Candida lipolytica</i>				
Ethanol	7.0	36	66	74
	9.5	28	44	47
Tetradecane	7.0	45	81	82
	9.5	38	58	62

and aa_3 content of the *C. lipolytica* mitochondria as compared with those of *S. cerevisiae* was still evident. This was true also for mitochondria isolated from both ethanol- and tetradecane-grown cells.

Mitochondrial ATPase

The pH profiles of the mitochondrial ATPase of *C. lipolytica* and *S. cerevisiae* have been described in a previous paper (Houghton *et al.* 1973). The specific activity of the *C. lipolytica* enzyme was never greater than $1.0 \mu\text{mol}/\text{min}/\text{mg}$ protein and, although comparable with that of *S. cerevisiae* in the pH range 6.0 to 7.0, it was much lower in the pH range 8.0 to 10.0 where the specific activity of the *S. cerevisiae* ATPase was 4.0 to $5.0 \mu\text{mol}/\text{min}/\text{mg}$ protein. The pH profile and specific activities of the ATPase of *S. cerevisiae* mitochondria were in good agreement with those of Kovac *et al.* (1968). The effect of varying the Mg^{2+} :ATP ratio at pH values 7.0 and 9.5 on the mitochondrial ATPase activities of *C. lipolytica* and *S. cerevisiae* cells grown on ethanol was examined. Both *S. cerevisiae* and *C. lipolytica* showed optimal ATPase activity at Mg^{2+} :ATP ratios of 1:1 and 1:2 at pH values 7.0 and 9.5 respectively. In *S. cerevisiae* the specific activity at pH 9.5 was still 3 to 4 times that at pH 7.0, even when both were assayed at their respective optimal Mg^{2+} :ATP ratios. Conversely, for *C. lipolytica* the specific activities at pH 7.0 and pH 9.5, again at their optimal Mg^{2+} :ATP ratios, were approximately the same.

Mitochondrial ATPase is characteristically inhibited by oligomycin in both mammalian and yeast mitochondria except that rather higher concentrations of oligomycin are required for comparable levels of inhibition of yeast ATPase. As shown previously (Houghton *et al.* 1973) *S. cerevisiae* ATPase was more resistant to inhibition by oligomycin when the activity was assayed at pH 7.0 than at pH 9.5 (Table 5). A completely opposite situation was found in *C. lipolytica* mitochondrial ATPase. Low concentrations of oligomycin in the range of $10 \mu\text{g}/\text{mg}$ protein gave only 40 % inhibition at pH 9.5 against 80 to 90 % for the *S. cerevisiae* activity. The picture was reversed at pH 7.0 in that the *C. lipolytica* ATPase was inhibited to 60 to 70 % but that of *S. cerevisiae* to about 40 %. Moreover, the inhibition of the *C. lipolytica* enzyme reached a plateau at high oligomycin concentrations (around $25 \mu\text{g}/\text{mg}$ protein) in both the pH 7.0 and 9.5 activities. The use of the F_1 inhibitor of Pullman & Monroy (1963) confirmed that the ATPase activities in the purified preparations from *S. cerevisiae* and *C. lipolytica* were essentially mitochondrial. In both, 70 to 80 % of the ATPase activities were inhibited by $150 \mu\text{g}$ F_1 inhibitor/mg protein at pH 7.0. The ATPase in *C. lipolytica* mitochondria isolated from cells grown on tetradecane was slightly more

sensitive to inhibition by oligomycin, at pH 7.0 and pH 9.5, than that in mitochondria obtained from ethanol-grown cells. Oligomycin at a concentration of 10 $\mu\text{g}/\text{mg}$ protein gave 80 % and 60 % inhibition respectively at pH 7.0 and pH 9.5 (Table 5).

DISCUSSION

The growth of *C. lipolytica* on even-numbered hydrocarbons (C_{10} to C_{16}) resulted in cells containing over 95 % of even-numbered fatty acids, predominantly $\text{C}_{18:1}$ and $\text{C}_{18:2}$. These results indicate chain elongation and desaturation of fatty acids. The fatty acids presumably arose from hydrocarbon oxidation, which has been suggested to be via the primary alcohol and then by dehydrogenation to the corresponding acid (Klug & Markovetz, 1970).

The presence of high amounts of even-numbered fatty acids in *C. lipolytica* cells grown on odd-numbered hydrocarbons, particularly C_{11} and C_{13} (Table 1), indicated *de novo* synthesis and hence a possible cell requirement for even-chain fatty acids. On the other hand, growth in C_{15} hydrocarbon gave cells with about 20 % of even-chain fatty acids and over 70 % of the unusual fatty acid, $\text{C}_{17:1}$.

A predominance of cell fatty acid corresponding in chain length to the alkane substrate utilized has recently been reported in *Candida tropicalis* by Hug & Fiechter (1973). These results differed from our observations, in which a predominance of cell fatty acid of longer chain length than the alkane substrate used was observed. In the present studies the cells were harvested at the end of exponential phase or early stationary phase and a lower substrate concentration was used (0.1 to 0.4 %, v/v) than in the experiments of Hug & Fiechter (1973) (1 %, v/v). It would appear, therefore, that the early steps of hydrocarbon utilization and fatty-acid synthesis were studied by Hug & Fiechter (1973).

We have emphasized that the major fatty acids of *Candida*, namely $\text{C}_{18:1}$ and $\text{C}_{18:2}$, were different from those characteristic of *Saccharomyces*, $\text{C}_{16:1}$ and $\text{C}_{18:1}$. These differences may be important in conferring the structural and functional properties on the cell membranes necessary to assimilate the lipophilic hydrocarbon substrates. It is noteworthy, in this respect, that electron micrographs of yeast cells utilizing hydrocarbons have shown extensive invaginations of the plasma membrane (Ludvik, Munk & Dostalek, 1968).

The mitochondrial membrane-bound enzymes (NADH oxidase, succinate oxidase and succinate dehydrogenase) of *C. lipolytica* cells grown on ethanol showed significantly higher specific activities and lower transition temperatures when compared with the corresponding enzymes of *S. cerevisiae* (Table 3). The differences in specific activities of the respiratory-chain enzymes were supported by analysis of cytochrome spectra, which showed relatively high concentrations of cytochromes, particularly cytochromes *aa₃* and *b*, in the *Candida* cells and mitochondria (Table 4). The lower transition temperatures observed for the respiratory-chain enzymes of ethanol-grown *Candida* cells is readily explicable in view of the recent work on the membranes of *Mycoplasma laidlawii* (Steim *et al.* 1969; Engelman, 1971; McElhaney, De Gier & van der Neut-Kok, 1973) and *Escherichia coli* (Wilson, Rose & Fox, 1970; Overath, Schairer & Stoffel, 1970; Esfahani *et al.* 1971). These studies have shown that breaks in Arrhenius plots of membrane-associated enzyme activity may be correlated with the degree of unsaturation of the membrane lipids: the greater the degree of unsaturation, the lower the transition temperature. A similar phenomenon has been reported in mitochondria isolated from aerobic and anaerobic cells of *S. cerevisiae* (Ainsworth *et al.* 1972; Watson *et al.* 1973*a*). More recent studies in this laboratory on a fatty-acid auxotroph of *S. cerevisiae* have further shown a close correlation between specific activities, as well as transition temperatures, of mitochondrial membrane-bound enzymes

and the degree of unsaturation of the membrane lipids (K. Watson, R. L. Houghton, E. Bertoli and D. E. Griffiths, unpublished).

A complicated picture was seen in a comparison of the mitochondrial ATPase of *C. lipolytica* and *S. cerevisiae*. The major observations were the much lower specific activity of the *Candida* ATPase and marked differences in pH profile and sensitivity to oligomycin. These observations may be an expression of distinct lipid-protein interactions within the mitochondrial membranes. It has been long established that phospholipids play a key part in the proper functioning of mitochondrial respiratory enzymes (Fleischer, Brierley, Klouwen & Slautterback, 1962; Green & Tzagoloff, 1966) and mitochondrial ATPase (Kagawa & Racker, 1966). More recent reconstitution studies have confirmed that phospholipids have a profound effect not only on the mitochondrial ATPase (Pitotti, Contessa, Dabenni-Sala & Bruni, 1972; Swanljung, Frigeri, Ohlson & Ernster, 1973) but also on the microsomal (Na⁺-K⁺)-ATPase activity (Taniguchi & Iida, 1972; Stahl, 1973).

The ability to manipulate the lipid composition of *C. lipolytica* and *S. cerevisiae* allows the design of experiments to investigate possible relationships between membrane lipid composition and enzyme activity. The incorporation of fatty acids not normally found in mitochondria, e.g. C_{17:1}, by growth of *C. lipolytica* on odd-numbered *n*-alkanes, is thus of interest.

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