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The Manipulation of Cellular Cytochrome and Lipid Composition in a Haem Mutant of *Saccharomyces cerevisiae*

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1. The *ole-3* mutant of *Saccharomyces cerevisiae* has an early lesion in the pathway of porphyrin biosynthesis. 2. This results in the loss of all haem-containing enzymes, including the mitochondrial cytochromes, and prevents the synthesis of components whose formation requires haem-containing enzymes, including unsaturated fatty acids, ergosterol and methionine. 3. The pleiotropic effects of the primary lesion are reversed by growing mutant *ole-3* aerobically in the presence of intermediates of the porphyrin-biosynthetic pathway, and the present work reports the degree of manipulation of lipid and respiratory-cytochrome composition. 4. Supplements of δ-aminolaevulinate in the range 0.5–500 mg/l result in a progressive increase in the cellular content of unsaturated fatty acids and respiratory cytochromes, cause the replacement of lanosterol and squalene by ergosterol, and an increase in total sterol content. 5. Haematoporphyrin and protoporphyrin IX have similar but less extensive effects on cellular composition, whereas haematin allows unsaturated fatty acid synthesis and some sterol synthesis, but has no effect on the formation of respiratory cytochromes. 6. These results suggest that growth of the organism in the presence of defined amounts of δ-aminolaevulinate will be useful in the investigation of the role of lipids and cytochromes in the function and assembly of mitochondrial membranes.

Resnick & Mortimer (1966) isolated a number of oleic acid auxotrophs of *Saccharomyces cerevisiae*, and showed that one group of these, mutants *ole-2*, *ole-3* and *ole-4*, is simultaneously respiratory-deficient. Subsequently, Bard (1972) showed that these mutants are nystatin-resistant, blocked in sterol synthesis, and require ergosterol and methionine for optimal growth on defined media. Bard *et al.* (1974) reported that all three mutants accumulate lanosterol and squalene, lack detectable cytochrome pigments and have negligible respiratory activity. The lipid growth requirements of strains *ole-2* and *ole-3* are abolished by intermediates of porphyrin biosynthesis, certain of which also allow cytochrome synthesis and antimycin A-sensitive respiratory activity, indicating that the primary defect in the mutants is a lesion in the porphyrin-biosynthetic pathway (Bard *et al.*, 1974). Thus the pleiotropic lesions in the mutants result from the loss of essential coenzymes that are the end products of the porphyrin pathway. Woods *et al.* (1975) have shown that mutant *ole-3* is deficient in δ-aminolaevulinate synthase (EC 2.3.1.37).

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The present work details the effects of defined concentrations of intermediates in porphyrin biosynthesis on the lipid and cytochrome composition of mutant *ole-3*. The results demonstrate that extensive manipulation of the cellular content of unsaturated fatty acids, sterols and the activity of mitochondrial respiratory enzymes is possible. In consequence this mutant can be utilized to probe the role of lipids and haem prosthetic groups in the function and assembly of membrane-bound mitochondrial enzymes.

Experimental

Growth media

Liquid growth media contained Difco yeast extract (Difco Laboratories, West Molesey, Surrey, U.K.) (10 g/litre), the *Saccharomyces* salts mixture described by Wallace *et al.* (1968) [$(NH_4)_2SO_4$ (1.2 g/litre); NaCl (0.5 g/litre); $CaCl_2$ (0.1 g/litre; $MgCl_2$ (0.7 g/litre); KH_2PO_4 (1.0 g/litre); $FeCl_3$ (3 mg/litre)], and a source of energy, either glucose (50 g/litre) or ethanol (10 g/litre). The pH of all culture media was adjusted to 5.4 before autoclaving at 0.1 MPa for 15 min. Unsaturated fatty acids were added as Tween 80 (10 g/litre). The porphyrin

supplements protoporphyrin IX, haematoporphyrin and haemin were sterilized by dissolving in ethanol in a sterile tube at a concentration of 20mg/ml. δ-Aminolaevulinate was dissolved in ethanol at 20–500mg/l, and then added to the growth media at the appropriate concentration, such that the ethanol concentration of glucose media did not exceed 1g/litre. Solid media for plates contained Difco yeast extract (2g/litre), *Saccharomyces* salts, glucose (20g/litre), Difco agar (20g/litre), with or without Tween 80 (10g/litre). These plates were used to assay contaminant and revertant cells. Results of experiments in which revertants exceed 1% of total cells were discarded. Stock solid slopes for the maintenance of yeast cultures contained Difco yeast extract (10g/litre), bacteriological peptone (5g/litre, Oxoid, Basingstoke, Hants., U.K.), glucose (50g/litre), Tween 80 (10g/litre), Difco agar (20g/litre) and *Saccharomyces* salts. With the *ole-3* mutant better growth was obtained by including ergosterol (10mg/litre) in the stock slope medium, but the latter was not essential.

Yeast strains

The strains of *S. cerevisiae* used in these studies are the *ole-3* mutant and the wild-type strain S288C from which it was derived. These strains have been described previously (Resnick & Mortimer, 1966; Bard, 1972; Bard *et al.*, 1974).

Culture conditions

All cultures were grown aerobically at 28°C on a rotary shaker. Sufficient inoculum was added to give an initial cell density of 0.5–1.0mg dry wt. of cells/litre in glucose medium and 1–5mg dry wt. of cells/litre in ethanol medium, from a starter culture grown overnight on glucose medium plus δ-aminolaevulinate (50mg/litre). All cultures were harvested at early stationary phase.

Estimation of cell growth yields

Cell growth yields were determined volumetrically by centrifuging at 500*g* for 5min a sample of cells in calibrated thick-walled graduated glass capillary tubes. For the determination of the doubling times of cell growth, cultures were grown in fluted 250ml conical flasks containing 75ml of culture medium with a side-arm attachment. Growth was determined by absorbance in an EEL colorimeter (with a neutral filter) which had been calibrated from the direct weighing of dried-cell samples. The log of growth extent was plotted against time, and the doubling times were calculated from the linear part of the graph.

Measurement of respiration

Respiration was measured in a closed Clark oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) at 30°C. Cells were harvested, washed in potassium phosphate buffer (50mM, pH7.0) and resuspended at a concentration of 15–20mg dry wt. of cells/ml of buffer. Whole cells (1–5mg dry wt./ml) were suspended in 3.0ml of potassium buffer (50mM, pH7.4), containing glucose (2mM) or ethanol (55mM), in both the presence and the absence of antimycin A (5mg/l) as a specific inhibitor of mitochondrial respiration.

Whole-cell cytochrome spectra

After harvesting and washing, a few grains of sodium dithionite were added to a cell sample (15–20mg dry wt. of cells/ml) in a 3ml cuvette (10mm path length). Spectra were determined in an SP. 1800 double-beam spectrophotometer by reading against a reference cuvette containing a starch gel adjusted to an opacity similar to that of the yeast cell suspensions (Clark-Walker & Linnane, 1967).

Lipid analyses

Total fatty acids in cells and mitochondria were analysed essentially as described by Proudlock *et al.* (1971). Cellular and mitochondrial sterol analyses were performed on the neutral-lipid extracts by g.l.c. on a 1.5m×6.4mm glass column with 1% SE30 absorbed on Gas-Chrom Q (100–120 mesh) at 240°C. The sterols were identified by their retention times on the column relative to cholestan-3β-ol, by using a standard mixture of squalene, cholestan-3β-ol, ergosterol and lanosterol. To determine the total sterol content of cells or mitochondria, a known weight of cholestan-3β-ol was added before saponification. In most experiments sterol extracts were analysed within 24h, as some decomposition of ergosterol occurs during storage, even at −20°C under N_2 in the dark. If sterols were left for longer than 24h, they were purified by t.l.c. before separation by g.l.c. T.l.c. of the neutral-lipid extract was carried out on 250mm×150mm plates by using 0.5mm-thick silica gel G (Merck, Darmstadt, Germany). The plates were activated before use by heating in an oven at 110°C for 1h. The solvent system used was chloroform, and the sterols on the developed chromatograms were detected either by charring with 40% (v/v) H_2SO_4, or by developing with Rhodamine-6G (0.1%, w/v) in acetone, after which the sterols were detected under u.v. light and eluted into diethyl ether.

Other methods

Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard

over the range 25–200 μg of protein. Ethanol concentrations were assayed by a modification of the method of Cooper (1971). A 1 ml culture sample was centrifuged, and 0.2 ml of the supernatant transferred to 3 ml of propan-1-ol (1 mg/ml). A 1–5 μl sample was analysed by g.l.c., by using a 1.5 m × 6.4 mm Poropak Q column (80–100 mesh) at 150°C with N_2 as carrier at a flow rate of 50–60 ml/min. Authentic samples of methanol, acetaldehyde, ethanol, acetone and propan-1-ol were used as reference samples, and the ethanol concentration was estimated by triangulation, with propan-1-ol as the internal standard.

Materials

δ-Aminolaevulinic acid, haematoporphyrin, haemin, antimycin, lanosterol and ergosterol were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Protoporphyrin IX was from Calbiochem, San Diego, CA, U.S.A. Yeast extract was obtained from Difco Laboratories, Detroit, MI, U.S.A., and bacteriological peptone from Oxoid, London E.C.4, U.K. Tween 80 was obtained from BDH Chemicals, Poole, Dorset, U.K. Poly(ethylene glycol) adipate and SE 30 were from Applied Science Laboratories, State College, PA, U.S.A. Dr. L. J. Goad, University of Liverpool, provided the authentic samples of squalene and cholestan-3β-ol. All other reagents used were of the highest chemical grade available.

Results

Growth yields and doubling times of strains S288C and ole-3

The potential of the *ole-3* mutant as an experimental system for studying mitochondrial assembly has been discussed by Bard *et al.* (1974), and a detailed analysis of its growth characteristics, and the extent to which its lipids and cytochromes could be manipulated, was undertaken. The growth yields and doubling times of mutant *ole-3* and wild-type strain S2886 grown on glucose and ethanol media supplemented with various porphyrin intermediates are shown in Tables 1 and 2 respectively. An increase in δ-aminolaevulinate concentration in the range 0–500 mg/l in either glucose or ethanol medium causes a progressive decrease in the doubling time of mutant *ole-3*, until at the highest concentration of δ-aminolaevulinate tested the mutant grows at a similar rate to the wild-type, S288C. A supplement of δ-aminolaevulinate at a concentration of 0.5 mg/l does not stimulate growth on glucose, but at 5 mg/l the doubling time is decreased to 6 h, and decreases to 2 h with supplements of 500 mg/l. The effects of δ-aminolaevulinate on the growth of mutant *ole-3* on ethanol medium parallel those observed with glucose-cultured cells, but markedly higher supplements of δ-aminolaevulinate (in excess of 10 mg/l) are required to obtain a significant decrease in doubling times and cell growth yields. Addition of exogenous δ-aminolaevulinate to wild-type cells also stimulates growth on both glucose and ethanol media, indicating that it is possible to modify cell physiology by boosting the supply of porphyrin intermediate,

Table 1. *Growth yields and doubling times of strains S288C and ole-3 on glucose medium supplemented with porphyrin intermediates*

The results represent the average values for at least four determinations for cells grown on glucose media for 48 h. Cell growth yields and doubling times are calculated from the growth curves as described in the Experimental section.

Strain	Porphyrin supplement	Concn. (mg/l)	Growth (mg dry wt./ml)	Doubling time (h)
S288C	None	0	4.2	1.6
	δ-Aminolaevulinate	50	6.1	1.2
ole-3	None	0	0.2	>24
	δ-Aminolaevulinate	0.5	0.2	22.8
		2.0	0.8	6.6
		5.0	1.1	6.0
		10	1.4	5.5
		20	2.2	5.3
		50	3.4	4.2
		150	4.0	3.3
		500	6.6	2.0
	Haematoporphyrin	20	3.2	2.1
	Haemin	10	2.9	2.6
	Protoporphyrin IX	50	2.7	3.0

Table 2. *Growth yields and doubling times of strain S288C and ole-3 on ethanol medium supplemented with porphyrin intermediates*

Cells were grown on ethanol medium (10 g/l) for 72 h and data represent the average values of at least three experiments. Other conditions are as in Table 1.

Strain	Porphyrin supplement	Concn. (mg/l)	Growth (mg dry wt./ml)	Doubling time (h)
S288C	None	0	3.4	4.4
	δ-Aminolaevulinate	50	4.4	3.6
ole-3	None	0	0.2	>24.0
	δ-Aminolaevulinate	0.5	0.4	23.9
		10	0.8	10.4
		50	2.2	9.2
		100	2.7	7.3
		150	2.8	6.8
		200	3.5	6.5
		500	4.0	5.3
	Haematoporphyrin	20	3.2	3.9
	Haemin	10	0.2	>24.0
	Protoporphyrin IX	50	2.8	4.2

Table 3. *Whole-cell fatty acid composition of S288C and ole-3 cells grown on porphyrin-supplemented glucose medium*

Cells were grown on glucose media with various porphyrin supplements into late exponential phase (24h), and lipids were analysed as described in the Experimental section. The results given are those of typical experiments.

Strain	Porphyrin supplement	Concn. (mg/l)	Fatty acid composition (%, w/w)								Total (%)		
			$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{14:1}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{10:0}$–$C_{14:0}$	$C_{16:0}$+$C_{18:0}$	$C_{16:1}$+$C_{18:1}$
S288C	None	0	0.6	2.5	3.3	0.6	21.0	32.0	3.0	37.0	7.0	24.0	69.0
	δ-Aminolaevulinate	50	0.4	2.1	2.0	0.5	7.8	48.3	5.2	33.7	5.0	13.0	82.0
ole-3	None	0	4.3	24.0	6.5	25.5	23.4	2.6	8.0	5.7	60.3	31.4	8.3
	δ-Aminolaevulinate	0.5	3.9	25.5	6.6	20.0	22.9	4.6	7.3	9.5	56.0	29.9	14.1
		2.0	1.3	15.0	5.1	8.1	23.1	26.1	7.3	14.0	29.5	30.4	40.1
		5.0	2.2	11.3	4.6	7.9	18.7	26.5	4.8	24.0	26.0	23.5	50.5
		20	1.4	10.2	3.3	7.6	14.6	30.1	3.6	30.2	22.5	18.2	60.3
		50	0.4	8.0	1.1	6.5	8.2	41.0	2.5	32.3	16.0	10.7	73.3
		150	0.5	4.4	1.0	2.0	8.4	44.1	2.6	37.0	7.9	11.0	81.1
		500	0.3	0.5	0.6	0.6	7.9	48.0	3.1	39.0	2.0	11.0	87.0
	Haematoporphyrin	20	6.3	4.6	2.6	1.1	23.4	30.5	5.3	22.9	17.9	28.7	53.4
	Haemin	10	2.5	3.2	2.5	1.1	21.8	35.0	3.7	30.2	9.3	25.5	65.2
	Protoporphyrin IX	50	2.0	3.6	2.1	1.0	21.3	35.5	5.5	29.0	8.7	27.8	64.5

Table 4. *Whole-cell fatty acid composition of S288C and ole-3 cells grown on porphyrin supplement ethanol medium*

The cultures were grown in early stationary phase (48h) on ethanol medium. Other conditions were as in Table 3. The results are of typical experiments.

Strain	Porphyrin supplement	Concn. (mg/l)	Fatty acid composition (%, w/w)								Totals (%)		
			$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{10:0}$–$C_{14:0}$	$C_{16:0}$+$C_{18:0}$	$C_{16:1}$+$C_{18:1}$
S288C	None	0	0	1.1	2.0	2.6	12.2	38.8	13.0	30.3	5.7	25.2	69.1
	δ-Aminolaevulinate	50	0	0.7	1.1	2.1	10.6	37.4	3.7	44.4	3.9	14.3	81.8
ole-3	None	0	16.2	1.9	5.5	8.4	33.6	16.3	12.0	6.0	32.0	45.6	22.3
	δ-Aminolaevulinate	0.5	11.1	3.4	5.0	6.6	33.0	21.3	11.7	8.0	26.0	44.7	29.3
		10	8.2	6.2	3.4	1.5	24.9	33.9	7.0	15.0	19.2	31.9	48.9
		20	5.1	3.3	1.1	0.3	15.0	34.0	6.5	32.7	9.8	21.5	68.7
		50	4.0	3.4	0.6	0.2	9.2	40.1	6.0	36.2	8.5	15.2	76.3
		100	3.3	1.2	1.7	0	9.1	40.7	6.0	38.0	6.2	15.1	78.7
		500	1.0	0.2	0.4	0	8.2	42.5	5.9	37.8	5.6	14.7	80.3
	Haematoporphyrin	20	5.6	2.4	2.0	0	23.0	32.0	6.4	28.6	10.0	29.4	60.6
	Haemin	10	17.3	2.4	1.5	6.6	32.4	17.0	10.9	11.9	27.8	43.3	28.9
	Protoporphyrin IX	50	2.6	0.4	1.3	0	13.0	33.5	5.4	43.8	4.3	18.4	77.3

Porphyrin intermediates such as haematoporphyrin (20mg/l) and protoporphyrin IV (50mg/l) stimulate growth on both substrates, and haemin (10mg/l) allows growth of the mutant on glucose medium, but not on ethanol medium. Haemin reverses the lipid lesion in mutant *ole-3*, but does not enable the synthesis of significant amounts of the mitochondrial respiratory cytochromes needed for growth on oxidizable substrates.

The inefficient use of the oxidizable substrates for energy production and the lower cell growth yields obtained at low concentrations of δ-aminolaevulinate supplements on ethanol media suggest that mitochondrial functions could be impaired in mutant *ole-3*. This phenomenon is further explored in the following paper (Astin & Haslam, 1977).

Manipulation of whole-cell lipid composition in strains ole-3 and S288C

Fatty acid composition of cells supplemented with porphyrin intermediates. The fatty acid compositions of S288C and mutant *ole-3* cells grown aerobically on glucose or ethanol media containing porphyrin supplements are shown in Tables 3 and 4. In the absence of supplements, the oleic acid plus palmitoleic acid content of *ole-3* cells declined to 8% of the total fatty acid content, with a corresponding increase in palmitic acid ($C_{16:0}$) and stearic acid ($C_{18:0}$) and an accumulation of the short-chain fatty acids $C_{10:0}$ to $C_{14:1}$. The composition of these depleted cells resembled that of anaerobically grown prototropic strains of *S. cerevisiae* (Meyer & Bloch, 1963; Jollow *et al.*, 1968), although there are higher contents of short-chain fatty acids, and particularly $C_{14:1}$ is detectable in large quantities in the mutant.

Supplementation with δ-aminolaevulinate at 0.5mg/l repairs the lesion in unsaturated fatty acid synthesis to some extent, but growth is not detectable. As the concentration of δ-aminolaevulinate is increased from 2 to 500mg/ml, the unsaturated fatty acid contents increase from 40 to 87% of total fatty acid. The maximum change in fatty acid composition occurs in palmitoleic acid ($C_{16:1}$), and is accompanied by a large increase in oleic acid content; this is correlated with a fall in short-chain fatty acid contents to those found in the wild-type. With δ-aminolaevulinate supplements of 50mg/l, the unsaturated fatty acid contents in mutant *ole-3* are similar to those of the wild-type. When δ-aminolaevulinate (50mg/l) is included in the growth medium of strains S288C or *ole-3* at 500mg/l, unsaturated fatty acid is stimulated to reach 82 and 87% in the wild-type and mutant respectively. These values are significantly higher than the normal unsaturated fatty acid content of 69% found in the wild-type strain.

Haemin, haematoporphyrin and protophyrin IX alleviate the unsaturated fatty acid requirement of mutant *ole-3* grown on glucose medium, but not as effectively as does δ-aminolaevulinate, and unsaturated fatty acid contents remain less than those found in the wild-type. It is difficult to test higher concentrations of these porphyrins, owing to their very low solubility in the medium. The data obtained with cells grown on ethanol as sole carbon source with various concentrations of porphyrin supplements are presented in Table 4. Here the cultures were grown for 48h to early stationary phase. The fatty acid composition changes in a similar manner to that in the glucose-grown cells, with a δ-aminolaevulinate concentration of 10mg/l causing a significant stimulation of unsaturated fatty acid synthesis, and a gradual increase to 80% with supplements of δ-aminolaevulinate of 500mg/l. One significant difference between ethanol-grown cells and glucose-grown cells is that unsaturated fatty acid contents decline to a minimum of 22% on ethanol medium as compared with 8% on glucose medium, when δ-aminolaevulinate is absent. This phenomenon has been observed previously by Proudlock *et al.* (1971) in the *ole-1* mutant, where it is caused by a selective lesion in mitochondrial oxidative phosphorylation induced by unsaturated fatty acid depletion. Proudlock *et al.* (1971) showed that ethanol oxidation is uncoupled when unsaturated fatty acid contents in the mitochondria are decreased to approx. 20% of total fatty acid, and growth is consequently prevented on non-fermentable substrates. As observed in the glucose-grown cells, high concentrations of δ-aminolaevulinate lead to an excess production of unsaturated fatty acids in both mutant and wild-type cells, suggesting that a higher activity of the fatty acid desaturase has been induced. Of the other intermediates tested, only haematoporphyrin and protoporphyrin IX reverse the lesion in unsaturated fatty acid synthesis, and haemin has very little effect. The ethanol-grown cells do not appear to be impermeable to the latter intermediates, as they show in the reduced cytochrome spectra of whole cells.

If Tween 80 (10g/l) is added to growth medium of *ole-3* cells in the presence of δ-aminolaevulinate supplements, then unsaturated fatty acid contents are at least as high as those found in the wild-type on either glucose or ethanol media (70–80%).

Analysis of sterols in mutant ole-3 cells grown on glucose and ethanol media in the presence of porphyrin supplements. T.l.c. analysis of sterol extracts from mutant *ole-3* showed the presence of squalene and lanosterol with negligible amounts of ergosterol. The small quantity of ergosterol detected in the mutant comes from the growth medium, because the Difco yeast extract used in the medium contains approx. 20μg of ergosterol/g. When mutant *ole-3* is supplemented with ergosterol, significant quantities of the sterol are taken up by cells, though there does not appear to be any decrease in the contents of

Table 5. *Sterol content of S288C and ole-3 cells grown on glucose medium with porphyrin supplements*
The results represent the average sterol contents for at least three separate experiments. Cultures were grown to late exponential phase (24h) on glucose medium containing Tween 80 (10g/l). RRT is relative retention time, calculated relative to cholestan-3β-ol. RRT 0.52, squalene; RRT 1.12, zymosterol; RRT 1.21, ergosterol; RRT 1.30, unidentified sterol; RRT 1.40, 24(28)-dehydroergosterol; RRT 1.47, fecosterol; RRT 1.56, episterol; RRT 1.63, lanosterol.

			Amount of compound (mg/g dry wt. of cells)								
Strain	Porphyrin supplement	Concn. (mg/l)	RRT ... 0.52	1.12	1.21	1.30	1.40	1.47	1.56	1.63	Total sterol
S288C	None	0	0.27	0.05	1.51	—	—	0.22	0.35	—	2.13
	δ-Aminolaevulinate	50	0.49	—	3.51	—	—	0.22	1.19	—	4.92
ole-3	None	0	0.45	0.03	0.25	0.02	0.05	—	—	1.58	1.93
	δ-Aminolaevulinate	0.5	0.48	0.04	0.24	0.03	0.04	—	—	1.72	2.07
		2.0	0.46	0.02	0.27	0.03	0.10	—	—	1.61	2.03
		5.0	0.53	0.08	0.60	0.03	0.27	—	—	1.23	2.21
		20	0.60	0.07	0.85	0.06	0.51	—	—	0.97	2.46
		50	0.61	0.09	1.37	0.06	0.58	—	—	0.48	2.58
		150	0.61	0.10	1.42	0.07	0.65	—	—	0.59	2.83
		500	0.83	0.11	2.81	0.09	1.22	—	—	1.03	5.26
	Haematoporphyrin	20	0.43	—	0.25	0.47	—	0.12	—	1.83	2.67
	Haemin	10	0.36	—	0.30	0.06	—	0.09	—	2.19	2.64
	Protoporphyrin IX	50	1.01	1.48	1.80	—	—	—	—	1.01	4.29

Table 6. *Sterol content of S288C and ole-3 cells grown on ethanol medium plus porphyrin supplements*
Cells were grown and sterols determined as in Table 5. Results are the averages for at least three experiments.

			Amount of compound (mg/g dry weight of cells)						
Strain	Porphyrin supplement	Concn. (mg/l)	RRT ... 0.52	1.21	1.40	1.47	1.56	1.63	Total sterol
S288C	None	0	0.78	5.25	—	0.78	0.28	—	6.31
	δ-Aminolaevulinate	50	0.34	5.23	—	0.53	0.60	—	6.36
ole-3	None	0	1.98	0.27	0.60	—	—	0.09	0.96
	δ-Aminolaevulinate	0.5	1.60	0.29	0.72	—	—	0.36	1.37
		5	1.63	0.30	0.72	—	—	0.38	1.40
		10	0.69	1.43	0.87	—	—	0.65	2.95
		20	0.69	1.62	0.91	—	—	0.86	3.39
		50	0.67	2.83	0.93	—	—	0.89	4.65
		100	0.26	3.89	1.67	—	—	0.88	6.44
		500	1.20	6.54	2.40	—	—	0.76	9.70
	Haematoporphyrin	20	1.78	0.30	0.75	—	—	0.29	1.34
	Haemin	10	0.84	0.27	0.93	—	—	1.96	3.16
	Protoporphyrin IX	50	0.36	1.80	0.80	—	—	1.04	3.64

lanosterol or squalene. In contrast, there is a significant lowering of squalene and lanosterol contents correlated with an increase of ergosterol content when media supplemented with δ-aminolaevulinate are used for growth of mutant *ole-3*. δ-Aminolaevulinate therefore reverses the lesion in sterol synthesis.

Detailed analyses of sterols of S288C and *ole-3* cells grown on glucose and ethanol media supplemented with various concentrations of the porphyrin intermediates are presented in Tables 5 and 6. The results show that ergosterol is the major sterol found in the wild-type strain grown on either glucose or ethanol medium. When grown on glucose medium the wild-type cells also contain moderate amounts of squalene, fecosterol and episterol, but lanosterol is absent. When grown on ethanol medium the total sterol content increases 3-fold, and the proportion of ergosterol increases at the expense of episterol and zymosterol, but the relative proportion of squalene remains constant. Addition of δ-aminolaevulinate to ethanol medium does not increase ergosterol or total sterol contents in wild-type cells, but the total sterol content of cells grown in glucose medium in the

presence of δ-aminolaevulinate more than doubles, and reaches values similar to those of the ethanol-grown cells.

The *ole-3* mutant grows on glucose medium in the absence of δ-aminolaevulinate, provided that Tween 80 is added. The cells contain lanosterol as the major sterol, and the total sterol and squalene content is similar to that of wild-type cells. The mutant does not grow on ethanol medium, owing to the lack of respiratory cytochromes, but if an inoculum of *ole-3* cells that has previously been grown in the presence of δ-aminolaevulinate is then allowed to deplete its sterols in the absence of δ-aminolaevulinate, cells are obtained with total sterol contents that are 50% of normal. These cells contain mainly ergosterol plus 24(28)-dehydroergosterol and very large amounts of squalene, but the 4-desmethylsterols probably represent those that were present in the cells before depletion in the absence of δ-aminolaevulinate. When *ole-3* cells are grown in the presence of δ-aminolaevulinate supplements, the amounts of ergosterol and total sterol greatly increase as the concentration of δ-aminolaevulinate is raised from 0 to 500mg/l. On glucose medium, the ergosterol content increases 10-fold and the total sterol doubles, but the proportion of squalene remains constant. The increase in ergosterol content is largely at the expense of lanosterol in the presence of moderate supplements of δ-aminolaevulinate (5–50mg/l), but at the highest concentrations of δ-aminolaevulinate (150 and 500mg/l) the amounts of lanosterol and 24(28)-dehydroergosterol also increase. With growth ethanol medium there is a 20-fold increase in ergosterol, a 4-fold increase in 24(28)-dehydroergosterol, and a 10-fold increase in total sterol content of cells as δ-aminolaevulinate supplements are increased from 0 to 500mg/l. The increase in 4-desmethyl sterols is largely at the expense of squalene, but the proportion of lanosterol also decreases at the highest concentrations of δ-aminolaevulinate.

Protoporphyrin IX (50mg/l) is as effective as δ-aminolaevulinate in changing the sterol composition of *ole-3* cells grown on both glucose and ethanol media. Haematoporphyrin (20mg/l) and haematin (10mg/l) do not allow the synthesis of ergosterol, but haematin increases the amount of lanosterol particularly in ethanol-grown cells.

Manipulation of respiratory activity and cytochrome content by growing cells in the presence of porphyrin supplements

Tables 7 and 8 show the respiration of cells grown to late-exponential phase on glucose and ethanol media respectively. The mutant has negligible respiratory activity and lacks significant antimycin A-sensitive respiration, indicating the loss of the cytochromes of the terminal electron-transport chain. Significant antimycin-sensitive respiration is detected with supplements of δ-aminolaevulinate of 20mg/l on glucose medium, although small activities are observed at lower supplements (2–5mg/l). At concentrations of δ-aminolaevulinate above 50mg/l, the respiratory activity in mutant *ole-3* increases to rates in excess of those normally associated with glucose-grown wild-type cells. However, addition of δ-aminolaevulinate to cultures of strain S288C also increases the respiratory activity of the cells on glucose medium by more than 5-fold.

Table 7. *Respiration of wild-type and mutant cells grown on glucose medium with porphyrin supplements*
Respiration (ng-atoms of oxygen/min per mg dry wt. of cells) was measured as described in the Experimental section with glucose (55mM) as substrate at 30°C. Antimycin A was added where indicated at a concentration of 20mg/l. The results presented are means±S.D. for six determinations.

Strain	Porphyrin supplement	Concn. (mg/l)	Respiration −Antimycin A	Respiration +Antimycin A
S288C	None	0	69.0± 3.0	4.5±0.2
	δ-Aminolaevulinate	50	421 ±25.0	16.5±3.0
ole-3	δ-Aminolaevulinate	0	3.0± 1.0	2.1±0.3
		0.5	2.2± 1.0	2.0±0.3
		2.0	2.6± 0.9	2.0±0.5
		5.0	7.4± 1.0	1.3±0.6
		20	61.0±10	3.6±1.0
		50	120 ±12	5.4±1.6
		100	144 ±15	3.2±0.9
		200	197 ±25	3.5±0.8
		500	384 ±30	5.3±1.3
	Haematoporphyrin	20	55.0± 1.0	2.3±1.0
	Haemin	10	2.0± 0.5	1.1±0.2
	Protoporphyrin IX	50	47.0± 1.0	2.8±0.8

Table 8. *Respiration of wild-type and mutant cells grown on ethanol medium with porphyrin supplements*
Respiration (ng-atoms of oxygen/min per mg dry weight of cells) was measured as described in the Experimental section with ethanol (55mM) as substrate at 30°C. The results presented are means±S.D. of at least four determinations. Antimycin A is added where indicated to the incubations at a concentration of 20mg/l.

Strain	Porphyrin supplement	Concn. (mg/l)	Respiration −Antimycin A	Respiration +Antimycin A
S288C	None	0	257 ± 5.0	4.8±0.4
	δ-Aminolaevulinate	50	551 ±28.0	12.1±1.6
ole-3	None		2.0± 0.4	2.1±0.5
	δ-Aminolaevulinate	0.5	2.4± 0.1	1.4±0.3
		2.0	2.5± 0.3	2.0±0.7
		5.0	24.8± 1.2	2.2±0.4
		10	182 ± 4.0	1.8±0.5
		50	206 ±10.0	3.0±0.9
		100	224 ±24.2	3.3±1.0
		150	244 ±30	2.8±1.4
		200	263 ±21	3.2±1.0
		500	395 ±30	4.8±1.6
	Haematoporphyrin	20	116 ±10	2.4±0.2
	Haemin	10	2.4± 0.5	1.6±0.2
	Protoporphyrin IX	50	150 ±12	1.8±0.5

A similar graded response to δ-aminolaevulinate is observed in the respiratory rates of mutant *ole-3* grown with ethanol as energy source. A δ-aminolaevulinate supplement of 10mg/ml gives cells with high antimycin-sensitive respiration. Higher concentrations of δ-aminolaevulinate (50mg/l) produce even higher respiratory activities in both S288C and *ole-3* cells grown on ethanol media.

Protoporphyrin IX and haematoporphyrin enable the synthesis of respiratory enzymes in mutant *ole-3* cells in both glucose or ethanol media, but rates of respiration are somewhat lower than those obtained with δ-aminolaevulinate supplements. In contrast, haemin has no effect on respiratory activity.

Whole-cell reduced cytochrome spectra of S288C and ole-3 cells

Whole-cell reduced-cytochrome spectra between 480 and 640nm for *ole-3* cells grown as described in the above experiments are given in Fig. 1(*a*). In the absence of δ-aminolaevulinate, *ole-3* cells lack detectable cytochrome and porphyrin pigments. With δ-aminolaevulinate supplements of 20mg/l, the characteristic α-absorption bands of cytochromes $a+a_3$ (603nm), cytochrome *b* (559nm) and cytochrome *c* (550nm), and the β-bands of cytochromes *c* (519nm) and *b* (528nm), are evident. Although not shown in Fig. 1(*a*), smaller α and β absorption peaks of cytochromes *b* and *c* are first detectable in the cells grown with δ-aminolaevulinate supplements as low as 2–5mg/l in which respiratory activity is very low, but cytochromes $a+a_3$ are not detectable in these cells. The spectrum of *ole-3* cells grown on glucose in the presence of δ-aminolaevulinate at 20mg/l resembles that of S288C cells grown on glucose media (Fig. 1*b*), where the synthesis of particulate cytochromes is subject to catabolite repression. The cytochrome concentration in glucose-grown *ole-3* cells supplemented with δ-aminolaevulinate at 50mg/l is greater than in the wild-type glucose-grown cells, and the spectrum resembles that seen in de-repressed S288C cells grown on ethanol or that of δ-aminolaevulinate-supplemented wild-type cells grown on glucose. Therefore δ-aminolaevulinate overcomes the effects of catabolite repression on cytochrome synthesis in both strains S288C and *ole-3*, and it also produces extremely high concentrations of cytochromes in both organisms when they are grown on ethanol. It is not possible to carry out spectral analysis of cells grown with haematoporphyrin, protoporphyrin IX or haemin, because the porphyrins themselves which are accumulated by the cells mask the cytochrome absorption peaks.

Discussion

Reversal of the pleiotropic effects of the mutation in mutant ole-3 by different porphyrin intermediates

The natural porphyrin intermediates δ-aminolaevulinate and protoporphyrin IX can be used for the synthesis of all the haem-containing enzymes involved in fatty acid desaturation, sterol synthesis and the formation of cytochromes. However, the chemical intermediates haematoporphyrin and haemin have only limited effects on reversing the depletion of lipids and cytochromes. This is not because the

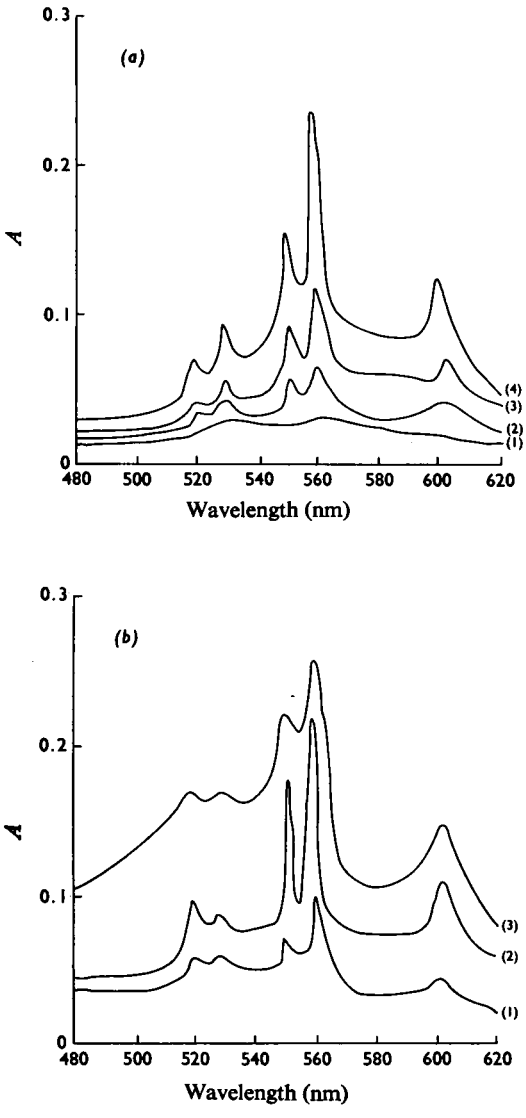

Fig. 1. *Whole-cell-reduced-cytochrome spectra of ole-3 and S288C cells*

Cells were grown aerobically at 28°C, and spectra determined as described in the Experimental section. (*a*): (1) *ole-3* cells on glucose medium; (2) *ole-3* cells on glucose medium supplemented with δ-aminolaevulinate (20mg/l); (3) *ole-3* cells on glucose medium supplemented with δ-aminolaevulinate (50mg/l); (4) *ole-3* cells on ethanol medium supplemented with δ-aminolaevulinate (500mg/l). (*b*): (1) S288C cells grown on glucose medium; (2) S288C cells grown on either ethanol medium or on glucose medium supplemented with δ-aminolaevulinate (50mg/l); (3) S288C cells grown on ethanol medium supplemented with δ-aminolaevulinate (50mg/l).

cells are impermeable to the latter intermediates, because cells grown with these compounds have visible absorption spectra characteristic of the added porphyrin compounds. The most probable explanation is that haematoporphyrin is a poor precursor of the prosthetic groups of cytochromes and other haem-containing enzymes, and that haemin is only significantly utilized to make the prosthetic group of the fatty acid desaturase, because yeast chemically modifies these compounds only very slowly.

Approximately 5 times as much δ-aminolaevulinate is required by mutant *ole-3* for growth on ethanol as on glucose medium, presumably reflecting the greater requirement for precursors of the respiratory cytochromes in order to grow on a non-fermentable substrate such as ethanol. As the δ-aminolaevulinate concentration in the growth medium is increased from 0 to 500mg/l, the unsaturated fatty acid content of *ole-3* cells increases progressively from 8 to 87% during growth on glucose medium and from 22 to 80% on ethanol medium. Supplements of δ-aminolaevulinate also increase unsaturated fatty acid contents in the wild-type to 82% of the total cellular fatty acids, suggesting that the synthesis of cytochrome b_5, which is an integral part of the fatty acid desaturase complex (Oshino *et al.*, 1966; Strittmatter *et al.*, 1974), may be controlled by the availability of the porphyrin prosthetic group.

The extensive manipulation of the total sterol content of *ole-3* cells is possible by growing the cells on either glucose or ethanol medium in the presence of increasing concentrations of δ-aminolaevulinate (0.5–500mg/l). On glucose medium, there is a progressive increase in total sterol content from 2 to 5mg/g dry wt. of cells. The proportion of ergosterol also rises from 13 to 54% of the total sterols, but is somewhat less than that found in wild-type cells (71%). When strain *ole-3* is grown in ethanol medium with δ-aminolaevulinate supplements, a greater range of manipulation of sterol content is possible, from 1 to 10mg of sterol/g dry wt. of cells, and at the highest supplements of δ-aminolaevulinate the proportion of ergosterol is similar to that found in the wild-type.

Control of sterol biosynthesis in yeast

Ethanol-grown wild-type and mutant cells contain larger quantities of total sterols than glucose-grown cells, indicating an effect of catabolite repression on the control of the pathway. The effect is reversed by δ-aminolaevulinate in both organisms. Cytochrome *P*-450 or a related haem prosthetic group has been indicated in the demethylation of lanosterol to C_{27} sterols (Wada *et al.*, 1969; Gibbons & Mitropoulos, 1973). Thus catabolite repression could limit sterol synthesis by restricting the supply of porphyrin precursors perhaps by inhibiting at the level of δ-aminolaevulinate synthase.

The sterol pathway is also subject to control by end-product inhibition at more than one point (Barton *et al.*, 1973; Fryberg *et al.*, 1975; Weete, 1973). The synthesis of mevalonate by hydroxymethylglutaryl-CoA reductase (EC 1.1.1.34) is thought to be inhibited by ergosterol, and the C_{24} methyltransferase (EC 2.1.1.41) enzyme has been suggested to be competitively inhibited as well as repressed by ergosterol (Thompson & Parks, 1974). The concept of feedback inhibition of the pathway by sterols is supported by the observation that large amounts of squalene are accumulated by *ole-3* cells that are extremely depleted in sterols by growth in ethanol medium in the absence of δ-aminolaevulinate. The present results also show that lanosterol is capable of exerting end-product inhibition, because the total sterol and squalene content of *ole-3* cells grown in the absence of δ-aminolaevulinate is normal.

It had previously been observed that the proportions of the individual sterols differ in cells grown on glucose and on ethanol media (Rogers *et al.*, 1974; Shimizu & Katsuki, 1975). At all concentrations of added δ-aminolaevulinate, the *ole-3* cells contain appreciable quantities of the intermediates of sterol synthesis. However, these differ, according to the energy source; glucose-grown cells contain squalene, lanosterol and zymosterol, whereas ethanol-grown cells lack zymosterol, accumulate much larger quantities of 24(28)-dehydroergosterol. but contain similar amounts of squalene and lanosterol. A possible explanation of these observations is that the C_{24} methyltransferase (EC 2.1.1.41) is inhibited and the 24(28) dehydrogenase an enzyme that converts 24(28)-dehydroergosterol into ergosterol, is activated under conditions of catabolite repression. However, an alternative explanation of the accumulation of 24(28)-dehydroergosterol by ethanol-grown cells is that the increased production of sterol intermediates induced by de-repression of an early step in the pathway causes the dehydrogenase to become rate-limiting in the conversion of sterol intermediates into ergosterol.

Manipulation of respiratory activity in ole-3 and S288C cells by growth in the presence of porphyrin supplements

Ole-3 cells completely lack antimycin-sensitive respiration, and have lost the cytochromes of the terminal electron-transport chain. By increasing δ-aminolaevulinate concentration from 2 to 500 mg/l, the respiratory activity of *ole-3* cells can be manipulated between 2 and 400 ng-atoms of oxygen/min per mg dry wt. of cells at 30°C. This system therefore affords an opportunity to study the assembly of a functional respiratory chain *in vivo*. The lower respiratory activities of yeast cells grown with glucose as the substrate can be attributed to catabolite repression, which affects the composition and oxidative ability of mitochondria (Ephrussi *et al.*, 1956; Wallace *et al.*, 1968; Lukins *et al.*, 1968). Cells grown on high concentrations of glucose develop very little respiratory activity, and contain smaller amounts of mitochondrial enzymes (Polakis & Bartley, 1965). Addition of δ-aminolaevulinate to glucose-grown S288C cultures completely removes all the effects of catabolite repression on cytochrome synthesis, and even stimulates respiration on ethanol media in both wild-type and mutant cells. The small amounts of ethanol added with the δ-aminolaevulinate do not affect the respiratory activity of the glucose-grown cells. The over-production of lipids and cytochromes obtained in the wild-type and mutant cells grown with δ-aminolaevulinate supplements suggests that δ-aminolaevulinate synthase is an important enzyme in the control of the metabolic pathway leading to all haem compounds in yeast. This enzyme is located within the inner mitochondrial membrane, and since it catalyses the first committed step in haem synthesis, one would expect it to be subject to control. The enzyme and its control has been the subject of many investigations in mammalian, bacterial and yeast systems (Beattie, 1971; Mahler & Lin, 1974; Sandy *et al.*, 1975). Its synthesis is inhibited by haem, an instance of end-product inhibition (Sinclair & Granick, 1975). In mammalian liver, the enzyme can be induced by a variety of chemical compounds (Sassa & Granick, 1970), and Beattie & Stuchell (1970) showed that in rat liver the induction of enhanced enzyme activities produces a significantly greater content of cytochromes *a*, a_3, *b*, *c* and c_1 in the mitochondria. This suggests that a possible relationship might exist between mitochondrial biogenesis and haem synthesis. This correlation has also been observed by Jayaraman *et al.* (1971) in studies on the yeast enzyme using inhibitors of mitochondrial and cytoplasmic protein synthesis. The latter studies also demonstrated that the enzyme is subject to catabolite repression and exhibits an oscillatory mode of synthesis. Sanders *et al.* (1973) used mutants of *S. cerevisiae* deficient in cytochrome biosynthesis to show that two unlinked nuclear genes, *cyd-1* and *cyc-4*, cause cells to be partially dependent on δ-aminolaevulinate for maximum cytochrome synthesis. Subsequent investigations by Woods *et al.* (1975) with the *ole-3* mutant demonstrated that δ-aminolaevulinate synthase activity of the mutant is less than 2% of that observed in the wild-type strain.

Order of synthesis of haem-containing enzymes

Our results clearly illustrate that, when the supply of haem precursors is manipulated in the *ole-3* mutant by varying the concentration of δ-aminolaevulinate supplements, the individual lesions are relieved in a certain order. Supplements of δ-aminolaevulinate as low as 0.5 mg/l partially relieve the

unsaturated fatty acid requirement; higher supplements (5–10mg/l) allow some respiratory activity, but only the highest supplements (500mg/l) restore the percentage of ergosterol to normal, although other sterols are formed in larger amounts than normal.

Use of the ole-3 mutant to study mitochondrial functions

The *ole-3* mutant allows us to manipulate contents of unsaturated fatty acids and sterols simultaneously, or of sterols alone if Tween 80 is added to the medium. It also enables the very extensive manipulation of respiratory activity and cytochrome content. This will facilitate the investigation of the effects of these lipids on the activity of the respiratory chain, and also the role played by lipids in the development and incorporation of cytochromes into newly formed mitochondrial membranes. Studies in a number of laboratories have shown direct correlation between respiratory functions and unsaturated fatty acid content (Haslam *et al.*, 1971, 1973; Rogers *et al.*, 1974; Marzuki *et al.*, 1975; Walenga & Lands, 1975) or sterol content (Parks & Starr, 1963; Thompson & Parks, 1974). Currently there is much interest in the assembly of the cytochromes in the mitochondrial membrane, particularly in cytochrome oxidase, whose biosynthesis resembles that of the inner mitochondrial membrane as a whole (for review see Schatz & Mason, 1974). The *ole-3* mutant will allow further investigation of the assembly process.

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