The Development of the Respiratory Chain of *Saccharomyces carlsbergensis* during Respiratory Adaptation

By T. G. CARTLEDGE and D. LLOYD

Department of Microbiology, University College, Cardiff CF1 3NR, U.K.

and MARIA ERECIŃSKA and B. CHANCE

Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa. 19104, U.S.A.

(Received 5 July 1972)

1. Subcellular fractionation of sphaeroplasts produced at different stages during the first 4 h of respiratory adaptation of anaerobically grown glucose-de-repressed *Saccharomyces carlsbergensis* gave mitochondrial fractions that contained all the detectable *c*- and *a*-type cytochromes. 2. The rates of cytochrome formation were studied; individual cytochromes were produced at different rates so as to give respiratory chains having widely differing cytochrome ratios. A CO-reacting haemoprotein other than cytochrome *a*₃ also increased throughout 8 h of respiratory adaptation. 3. Even after short periods of aeration, organisms contained mitochondria in which cytochrome–cytochrome interactions and the reaction of cytochrome *a*₃ with O₂ proceeded at rates almost as fast as in organelles from aerobically grown cells. 4. The technique of flow-flash photolysis enabled kinetic resolution of the reoxidation of cytochromes *a*₃ and *a* to be achieved and their individual contributions to extinction changes in the Soret region were assessed. The ratio cytochrome *a*₃/cytochrome *a* increased over the early stages of adaptation.

Changes in the haemoproteins of baker's yeast during the respiratory adaptation of anaerobically grown cells were originally observed with a micro-spectroscope (Chin, 1952, cited by Keilin, 1966; Ephrussi & Slonimski, 1950). The characteristic absorption bands of cytochromes '*a*₁' and '*b*₁' seen in the anaerobically grown organisms were replaced after aeration for 5 h by those of the cytochromes typically associated with respiratory chain of aerobic cells. The sequence of changes in the absorption bands of *b*- and *c*-type cytochromes during the progress of respiratory adaptation of *Saccharomyces cerevisiae* was also studied spectrophotometrically at the temperature of liquid nitrogen by Lindenmayer & Estabrook (1958), who observed the spectral transition from cytochrome *b*₁ (α -bands 557.5 and 551 nm) to cytochromes *b* (559 nm) and *c* (548 nm) and the appearance of the cytochrome *c*₁ α -band (554 nm). Although quantitative estimations of the cytochrome content of cells were not presented, no major increases in total haemoprotein was evident in these studies, and the possibilities of a precursor–product relationship between the cytochromes found in anaerobic cells and those produced during adaptation were discussed. Despite some evidence that extensive protein synthesis is not necessary for the development of respiratory activities (Bartley & Tustanoff, 1966), recent work in a number of laboratories clearly indicates that this is not the case and that both the increase in respiration rates (Rouslin & Schatz, 1969) and the

synthesis of cytochromes (Chen & Charalampous, 1969) are inhibited in the presence of a mixture of chloramphenicol and cycloheximide.

The spectra of anaerobically grown glucose-repressed *Saccharomyces carlsbergensis* resemble those of baker's yeast except that the α -band of cytochrome *a*₁ was not detected (Cartledge & Lloyd, 1972b). The kinetics of induction of cytochrome oxidase activity and cytochrome *a* (as measured in difference spectra at 605–630 nm; Chen & Charalampous, 1969) during the aeration of non-growing suspensions of anaerobically grown *S. carlsbergensis* were similar over a period of 4 h. In this paper we present a quantitative evaluation of the time-course of appearance of each of the cytochromes of the respiratory chain, a preliminary investigation of their subcellular distributions and a study of the kinetics of oxidation of the cytochromes during electron transport in mitochondria isolated from organisms at different stages of respiratory adaptation.

Materials and Methods

Saccharomyces carlsbergensis

Maintenance and growth. *Saccharomyces carlsbergensis* (N.C.Y.C. 74S) was maintained on agar slopes with 10% (w/v) glucose–malt extract (Wallace *et al.*, 1968).

Starter cultures were grown as previously described

(Cartledge & Lloyd, 1972*a*). The complex growth medium for anaerobic growth was as described by Cartledge & Lloyd (1972*b*). Batch cultures (12–18 litres) were grown in a glass vessel at 30°C and stirring rate was 400 rev./min. Before entry into the growth vessel high-purity N₂ was further purified as previously described (Cartledge & Lloyd, 1972*b*).

Adaptation of cells. Cells were harvested at a concentration of 6×10^7 cells/ml. These were washed once and resuspended in 2 litres of a medium containing (% w/v): glucose (0.4), yeast extract (0.2), CaCl₂ (0.033), KH₂PO₄ (0.9), MgSO₄ (0.05) and (NH₄)₂SO₄ (0.6). All transfer procedures were carried out under an atmosphere of N₂ and centrifugations were in sealed centrifuge pots. After 2 h at 30°C the cell suspension was added to 10 litres of the same medium, which had been aerated. Respiratory adaptation of the cells was stopped by the addition of chloramphenicol (1 mg/ml) and cycloheximide (0.1 mg/ml).

Harvesting of cells and preparation of sphaeroplasts. Cells were harvested and sphaeroplasts were prepared as described previously (Cartledge & Lloyd, 1972*a,b*).

Disruption of sphaeroplasts. Sphaeroplasts were disrupted as described previously (Cartledge & Lloyd, 1972*a*) except that 15 cycles of a Teflon hand homogenizer were used.

Preparation of whole homogenates and mitochondrial fractions. Partially digested cells, intact sphaeroplasts and nuclei were removed by centrifugation for 5 min at 1000*g* (*r*_{av} 7.6 cm) in the 8 × 50 ml rotor of a Sorvall RB2 centrifuge, and the resulting supernatant was decanted. The pellet was washed once and after centrifugation this supernatant was decanted. The two supernatants were mixed and the mixture was termed the whole homogenate.

A mitochondrial fraction was prepared by centrifugation of the whole homogenate for 10 min at 10000*g* (*r*_{av} 7.6 cm) in the 8 × 50 ml rotor of a Sorvall RB2 centrifuge. The pellet was washed three times and finally resuspended in a minimal volume, and this was termed the mitochondrial fraction. This fraction had no detectable endogenous respiration. The washings were combined to form the supernatant fraction.

Analytical methods

Cytochrome spectra. Total cytochrome contents (ascorbate + NNN'-tetramethyl-*p*-phenylenediamine-reduced minus-oxidized and Na₂S₂O₄-reduced minus-oxidized) of all three fractions were determined at room temperature in a split-beam spectrophotometer (Yang & Legallais, 1954) or at the temperature of liquid N₂ with this spectrophotometer used with the attachment described by Chance (1957). CO-difference spectra were obtained at room temperature after sparging cuvettes with CO for 2 min after reduction by the addition of Na₂S₂O₄.

It was not attempted to make the spectra at the

temperature of liquid N₂ quantitative as the values of ϵ for the individual cytochromes of yeast have not been determined at 77°K. To make quantitative the spectra recorded at room temperature the following wavelength pairs and reduced-minus-oxidized extinction coefficients were used: cytochrome *a*+*a*₃, 445–458, ϵ_{mM} 85 mm⁻¹·cm⁻¹ (Chance, 1953); cytochrome(s) *b*, 564–575, ϵ_{mM} 19 mm⁻¹·cm⁻¹; cytochromes *c* and *c*₁, 550–540, ϵ_{mM} 18 mm⁻¹·cm⁻¹ (Ohnishi *et al.*, 1967). In all the spectrophotometric experiments the mitochondria were suspended in the isolation buffer (0.25 M-sucrose – 2 mM-MgCl₂ – 10 mM-tris – HCl, pH 7.4).

Protein. Protein was assayed by the method of Lowry *et al.* (1951) with bovine serum albumin fraction V (Sigma Chemical Co., St. Louis, Mo., U.S.A.) used as a standard.

Kinetics of cytochrome oxidation

Reoxidation rates of cytochromes *b*, *c* and *c*₁ on mixing anaerobic mitochondrial suspensions with 17 μM-O₂ were observed in the regenerative flow apparatus of Chance (1954) with a dual-wavelength spectrophotometer (Chance, 1951). Ethanedol (20%, v/v) was included in reaction mixtures where reactions were studied at –3°C. The flow-flash photolysis apparatus used for measuring rates of reoxidation of cytochromes *a*₃ and *a* has been fully described by Chance & Erecińska (1971). Flash photolysis of CO-liganded cytochrome *a*₃ was achieved either in the absence of O₂ or 100 ms after mixing with 17 μM-O₂ during the flow of mitochondrial suspension through the observation tube. Over 95% photolysis was produced by a 220 mJ, 400 ns, 585 nm output of a cylindrical flashlamp-pumped Rhodamine 6-G-ethanol liquid dye laser (constructed by Mr. J. Bunkenberg). Time-shared measurements of the dual-wavelength extinction changes employed a 200 Hz chopping frequency and the technique of cycle selection (Chance & Erecińska, 1971). In many experiments kinetics of reoxidation of *b*- and *c*-type cytochromes were also initiated by the laser-induced dissociation of the cytochrome *a*₃-CO complex in the presence of O₂ and observed at appropriate wavelength pairs during the subsequent reoxidation of the electron-transport chains. When these experiments were conducted at room temperature (24°C), succinate-malonate mixtures were used to produce anaerobic mitochondrial suspensions with low rates of electron flux from substrate to O₂.

Results

Distribution of cytochromes and total protein

During the first 4 h of respiratory adaptation the percentage of total protein recovered in the mitochondrial fraction increased, but it decreased in the

Table 1. Distributions of total cytochrome contents of fractionated homogenates from *S. carlsbergensis* at different stages of respiratory adaptation

Conditions at time of harvesting Anaerobically grown Time of aeration (min)	Protein				b-type cytochromes				c-type cytochromes			
	Total amount (mg)		Total amount (nmol)		Total amount (nmol)		Total amount (nmol)		Total amount (nmol)		Total amount (nmol)	
	In whole homo- genate	In mito- chondrial fraction (A)	In whole homo- genate	In mito- chondrial fraction (A)	In whole homo- genate	In mito- chondrial fraction (A)	In whole homo- genate	In mito- chondrial fraction (A)	In whole homo- genate	In mito- chondrial fraction (A)	In whole homo- genate	In mito- chondrial fraction (A)
	Recovery (%)	Ratio A/B	Recovery (%)	Ratio A/B	Recovery (%)	Ratio A/B	Recovery (%)	Ratio A/B	Recovery (%)	Ratio A/B	Recovery (%)	Ratio A/B
Anaerobically grown	1060	800	0.37	103	60	5	15	0.33	33	<1.0	<3.0	<1.0
	1430	410	0.38	103	110	16.4	37	0.44	49	<8.0	<2.0	<8.0
	2020	494	0.36	93	173	—	82	—	—	—	<5.0	<5.0
	2390	864	0.53	104	93	65.5	102	0.64	176	54	42.5	<10
	3720	1310	0.46	111	458	238	372	0.64	133	238	298	<14
	2200	900	0.76	95	633	401	176	2.28	92	609	712	<14
Aerobically grown, glucose de- pressed	360	2280	0.22	106	575	354	366	2.8	84	660	460	3.3
	2100	229	0.13	95	388	169	366	0.46	127	321	87	0.32
	2100	426	0.25	102	237	86.5	82	1.05	72	156	98.4	<10
												66

last 4h (Table 1). The *c*- and *a*-type cytochromes detectable in difference spectra at room temperature were quantitatively recovered in mitochondrial fractions from homogenates prepared from organisms over the first 4h of adaptation; extensive release of these cytochromes in the supernatant fraction from cells adapted for 6 and 8h suggested that mitochondrial integrity is not preserved either before or during isolation after long-time adaptation under these conditions. During the first 6h of adaptation the ratio of total *b*-type cytochromes detected in the mitochondrial fraction to that in the supernatant fraction increases, but this ratio shows a marked decrease after 8h. The absorption maximum due to cytochrome *b* at 558nm produced on reduction with Na₂S₂O₄ progressively shifts to 561nm during adaptation. Reduction of some of the cytochrome *b* of whole homogenates (but not of mitochondrial fractions) was produced on the addition of 10mm-ascorbate + 1mm-*NNN'*-tetramethyl-*p*-phenylenediamine at early stages of adaptation (up to 2h).

Up to 6h adaptation both the cytochromes *b* and *c* (as measured from Na₂S₂O₄-reduced or ascorbate + *NNN'*-tetramethyl-*p*-phenylenediamine-reduced-oxidized spectra respectively at room temperature) of the mitochondrial fraction increase as a function of time; the rate of appearance of cytochrome *c* is only slightly greater than that of cytochrome *b* (Fig. 1*a*). After adaptation for 8h a marked decrease in the mitochondrial content of cytochrome *c* is accompanied by its accumulation in the supernatant fraction (Fig. 1*b*).

Cytochromes of mitochondrial fractions

To define absorption maxima due to *a*- and *c*-type cytochromes, difference spectra at 77°K were recorded with 10mm-ascorbate + 1mm-*NNN'*-tetramethyl-*p*-phenylenediamine as reductant (Fig. 2). A mitochondrial fraction from aerobically grown cells showed maxima at 599, 555 and 549nm corresponding to the α -bands of cytochromes *a* + *a*₃, *c*₁ and *c* and at 442 and 419nm corresponding to the γ -bands of *a*- and *c*-type cytochromes respectively. Mitochondrial fractions from anaerobically grown organisms show no absorption maxima on reduction under these conditions, apart from a small absorption band at 422nm. Adaptation for 10 or 30min leads to production of cytochrome *c* together with ascorbate + *NNN'*-tetramethyl-*p*-phenylenediamine-reducible cytochromes absorbing at between 547 and 563nm together with compounds with absorption maxima at between 600 and 650nm. The pigments responsible have not been further characterized. Progressive increases in the absorption due to *a*- and *c*-type cytochromes are seen during adaptation; cytochrome *c*₁ is evident after 4h, and both the 4h and 6h samples show increased content of cytochromes *a* + *a*₃, *c* and

Fig. 1. Cytochrome contents of mitochondrial fractions and supernatant fractions of homogenates prepared from sphaeroplasts of *S. carlsbergensis* at stages during respiratory adaptation

(a) Total $\text{Na}_2\text{S}_2\text{O}_4$ -reducible cytochrome *b* (▲) and ascorbate + *NNN'**N'*-tetramethyl-*p*-phenylenediamine-reducible cytochrome *c* (○) of the mitochondrial fractions. (b) Total $\text{Na}_2\text{S}_2\text{O}_4$ -reducible cytochrome *b* (▲) and total $\text{Na}_2\text{S}_2\text{O}_4$ -reducible cytochrome *c* (○) of the supernatant fractions. These values were measured from spectra obtained at room temperature by using the wavelength pairs and values of ϵ cited in the Materials and Methods section. Arrows indicate cytochrome contents of fractions from cells grown aerobically to the phase of glucose de-repression.

c_1 , compared with the mitochondrial fractions from aerobically grown glucose-de-repressed organisms. The contribution of cytochrome *b* (absorption maximum 560 nm, shoulder at 563 nm) is seen in $\text{Na}_2\text{S}_2\text{O}_4$ -reduced-oxidized spectra of aerobically grown cells (Fig. 3). The spectrum of the mitochondrial fraction of anaerobically grown organisms shows the presence of cytochrome '*b*₁', with absorption maxima at 551 and 556 nm, but no contribution from cytochrome '*a*₁' (attributed to cytochrome *c* peroxidase; Ishidate *et al.*, 1969a) has ever been distinguished in our strain of *S. carlsbergensis*

Fig. 2. Difference spectra of mitochondrial fractions from *S. carlsbergensis* at different stages in adaptation

Reduction was achieved by adding 10 mM-ascorbate + 1 mM-*NNN'**N'*-tetramethyl-*p*-phenylenediamine to the sample cuvette contents. Oxidation of the reference suspensions was by aeration; the cuvettes were then immersed in liquid N_2 and spectra recorded at 77°K. The pathlength throughout was 2 mm and spectral band width 1 nm. Protein concentrations were 15 mg/ml throughout, apart from that for the mitochondrial suspension of anaerobically grown cells, which was 10 mg/ml.

(Cartledge & Lloyd, 1972b). During the first 2 h of adaptation shifts in the absorption maxima due to *b*-type cytochromes occur until the aerobic pattern is attained.

Fig. 3. Difference spectra of mitochondrial fractions from *S. carlsbergensis* at different stages in adaptation

Reduction was achieved by adding excess of $\text{Na}_2\text{S}_2\text{O}_4$ to the sample cuvette contents. Oxidation of the reference suspensions was by aeration; the cuvettes were then immersed in liquid N_2 and spectra recorded at 77°K. The path length throughout was 2mm and spectral band width 1nm. Protein concentrations were 15mg/ml throughout, apart from that for the mitochondrial suspension of anaerobically grown cells, which was 10mg/ml.

CO difference spectra ($\text{Na}_2\text{S}_2\text{O}_4$ -reduced + CO minus $\text{Na}_2\text{S}_2\text{O}_4$ -reduced; Fig. 4) indicate the presence of a CO-reacting haemoprotein in the mitochondrial fraction from cells adapted for 10min (absorption maximum at 419nm, minimum at 439nm). Cytochrome *P*-450, which has been demonstrated in a similar spectrum of intact cells, may have a minor contribution at around 450nm. After adaptation for 1h the absorption maximum at 450nm is no longer apparent, but an increased absorption at 420nm (accompanied by a trough at 438nm and an α -band with a maximum at 570nm and minimum at 557nm) is evident. The mitochondrial content of these CO-reacting haemoprotein(s) increases throughout adaptation. The peak heights (419–432nm) were measured and, by using the value of $\epsilon = 170 \times 10^3$ for bacterial cytochrome *o* (Daniel, 1970), it was calculated that the mitochondrial content increased from 0.02 to 0.09nmol/mg of protein over an 8h period of adaptation. The α -bands at 534 and 570nm are also clearly indicative of an increase in haemoprotein other than cytochrome a_3 .

After 8h comparison of CO-difference spectra of whole homogenates (not shown) with those of mitochondria indicates that a CO-reacting haemoprotein with similar absorption characteristics has also accumulated in the supernatant fraction. After adaptation for 4h a trough at 444nm and a shoulder at 430nm indicate that cytochrome a_3 has become the major species contributing to this region of the spectrum.

Kinetics of reoxidation of cytochromes

The kinetics of reoxidation of cytochromes were investigated with the regenerative flow technique: typical traces obtained with the mitochondrial fraction from cells adapted for 6h are shown in Fig. 5. In this experiment a mitochondrial suspension containing 20% (v/v) ethanediol was allowed to respire in the presence of 10mM-ascorbate + 20 μM -NNN'-tetramethyl-*p*-phenylenediamine until anaerobiosis was attained. On mixing with 17 μM - O_2 the oxidation of cytochrome *c* (observed at 550–540nm) occurred very rapidly at room temperature ($t_{1/2} < 10\text{ms}$ at 24°C), but the half-time for the oxidation of cytochrome *b* (observed at 560–575nm) could be measured (90ms; Fig. 5a, trace 1). Observation at 566–575nm revealed that an extremely rapid reduction of another species of cytochrome *b* (b_T) occurred and was almost complete during the flow time ($t_{1/2} < 10\text{ms}$; Fig. 5b, trace 1). The reduction of cytochrome b_T is a general characteristic of the initiation of electron transport by O_2 pulses in anaerobic mitochondrial suspensions (Wilson *et al.*, 1971; Erecińska *et al.*, 1972; Chance, 1972).

At –3°C cytochrome *c* was oxidized with $t_{1/2} = 50\text{ms}$ (Fig. 5c, trace 1); the corresponding $t_{1/2}$ for cytochrome c_1 (554–540nm) was 70ms (Fig. 5d, trace 1).

Fig. 4. CO difference spectra of mitochondrial fractions from *S. carlsbergensis* at different stages in adaptation

Both sample and reference cuvettes contained mitochondrial suspensions reduced by $\text{Na}_2\text{S}_2\text{O}_4$. Base lines (dithionite-reduced minus dithionite-reduced) were recorded and showed no inflexions over the wavelength range studied. The sample cuvette contents were sparged with CO for 2 min before the difference spectra at room temperature were recorded. The path length throughout was 1 cm and spectral band width 1 nm. Protein concentrations (mg/ml) were as follows: 10 min-adapted cells, 7.9; 1 h-adapted cells, 9.0; 4 h-adapted cells, 9.7; 8 h-adapted cells, 4.4.

Cytochrome a_3 was observed by changes in extinction (445–458 nm) after the addition of $300 \mu\text{M}$ -CO (Fig. 5e). The technique of flow-flash photolysis (Chance & Erecińska, 1971) was used to dissociate the cytochrome a_3 -CO complex. In the absence of O_2 photolysis leads to an increase in extinction (Fig. 5f,

trace 1) and at -3°C the rate of recombination of CO with the reduced cytochrome a_3 thus liberated was negligible. When the laser was discharged 100 ms after mixing with $17 \mu\text{M}$ - O_2 , the reduced cytochrome a_3 was reoxidized so rapidly that the initial reaction (i.e. the decrease in extinction from the value achieved on anaerobic photolysis) did not appear on the trace, but the subsequent oxidation of cytochrome a was recorded with a $t_{\frac{1}{2}} = 15$ ms (Fig. 5f, trace 3). Increased time-resolution (not shown) revealed that the oxidation of cytochrome a_3 proceeded with $t_{\frac{1}{2}} < 7$ ms.

Kinetics of cytochrome reoxidation were studied at room temperature in mitochondrial fractions obtained at various stages of adaptation (Table 2). The respiratory substrate used to produce the anaerobic state was 6 mM-sodium succinate; 3 mM-sodium malonate was used to reduce the electron flux through the respiratory chain to minimize reaction rates in the reverse direction during the measurement of oxidation on pulsing with O_2 . The ability to oxidize succinate is not manifest in mitochondrial fractions from organisms adapted for 30 min or 1 h, and in these cases 10 mM-ascorbate + $20 \mu\text{M}$ -NNN'-tetramethyl-*p*-phenylenediamine was employed as reductant; the low concentration of cytochromes $a+a_3$ in these mitochondria made accurate assessment of their oxidation rates impossible. In all cases the oxidation rates are compatible with the sequence cytochromes a_3, a, c_1, c, b_{560} . These rates were all maximal after adaptation for 5–7 h. Biphasic reactions occurred for cytochromes c and c_1 only in mitochondrial preparations from cells adapted for up to 1 h, and occurred again in the 8 h-adapted sample. Flow-flash photolysis enabled kinetic resolution of the individual contributions of cytochromes a_3 and a to the total extinction change observed at 445–458 nm; thus it was possible to determine the relative ratios of these two cytochromes present at various times during respiratory adaptation (Fig. 6). In mitochondrial suspensions from aerobically grown, glucose-de-repressed cells and from cells adapted for 8 h equal amounts of cytochromes a and a_3 were observed, whereas the relative contribution of a_3 to the total extinction at 445–458 nm (calculated by using a value of 170 for the ϵ_{mM} cytochrome a_3 -CO minus cytochrome a_3) was only 39% after 3 h. The proportions of total a :' b ': c -type cytochromes in mitochondrial fractions from 1 h-adapted cells were 1:29:19, whereas these proportions were 1:14:19 after 6 h adaptation and 1:8:8 in mitochondrial fractions from aerobically grown glucose-de-repressed cells.

Discussion

Gentle mechanical disruption of sphaeroplasts of *S. carlsbergensis* (at any stage over the first 5 h of respiratory adaptation) releases a mitochondrial

Fig. 5. Kinetics of cytochrome reactions in mitochondria from *S. carlsbergensis* after respiratory adaptation for 6 h (a–d) Measurement of kinetics of *b*- and *c*-type cytochromes in the regenerative flow apparatus. (a) Trace 1, cytochrome *b* oxidation at 560–575 nm on mixing with O_2 at 24°C; trace 2, flow-velocity trace. (b) Trace 1, cytochrome *b* reduction at 566–575 nm on mixing with O_2 at 24°C; trace 2, flow-velocity trace. (c) Trace 1, cytochrome *c* oxidation at 550–540 nm on mixing with O_2 at –3°C; trace 2, flow-velocity trace. (d) Trace 1, cytochrome *c* reduction at 554–540 nm on mixing with O_2 at –3°C; trace 2, flow-velocity trace. Mitochondrial protein 5 mg/ml, respiratory substrate 10 mM-ascorbate + 20 μ M-*NNN'*-tetramethyl-*p*-phenylenediamine, 17 μ M- O_2 , path length 6 mm. (e–f) Kinetics of photolytic decomposition of CO-liganded cytochrome oxidase and the subsequent reactions with O_2 at –3°C. (e) Diagram of idealized trace indicating sequence of reactions producing changes of extinction in experiments shown in (f). Addition of ascorbate + *NNN'*-tetramethyl-*p*-phenylenediamine to a mitochondrial suspension gives a steady-state level of reduction of cytochromes *a* + *a*₃ corresponding to the trace at position (1). Attainment of anaerobiosis gives an increase in E (2) due to cytochrome reduction. Mixing with 300 μ M-CO leads to a 50% decrease in E (3) resulting from formation of reduced cytochrome *a*₃-CO complex. Flash photolysis dissociates this complex and restores E to the value (4) obtained before CO addition; in the absence of O_2 recombination of cytochrome *a*₃ and CO occurs (5). Mixing with O_2 100 ms before photolysis allows both cytochromes *a*₃ and *a* to become oxidized after the flash, but resolution of the very fast initial reaction is not attained. Better time-resolution indicates an increase in E (6), resulting from the dissociation of the reduced cytochrome *a*₃-CO complex, followed by oxidation of released reduced cytochrome *a*₃, during which E falls rapidly to a value (7) corresponding to that before photolysis. This reaction is followed by a further slower decrease in E (8), which corresponds to that expected for the reoxidation of reduced cytochrome *a*. (f) Trace 1, changes in E produced during anaerobic photolysis; trace 2, flow-velocity trace (downward displacement indicates period of mixing with oxygenated buffer); trace 3, changes in E produced on mixing with oxygenated buffer followed by photolysis. Increased time-resolution was employed to record the initial reaction after photolysis.

Table 2. Kinetics of cytochrome reactions in mitochondria from *S. carlsbergensis* at different stages in respiratory adaptation

Reactions were observed at the wavelength pairs indicated in Fig. 5; respiratory substrates were 10 mM-ascorbate + 20 μ M-NNN'N'-tetramethyl-*p*-phenylenediamine in experiments with mitochondria from cells adapted for 30 min, 1 h and 6 h and with those from aerobically grown organisms. In all other cases substrate was 6 mM-sodium succinate + 3 mM-sodium malonate. Temperature of incubation was 24°C except for values shown in parentheses, where experiments were at -3°C in the presence of 20% (v/v) ethanol.

Cytochrome	Half times (ms) for oxidation in mitochondrial suspensions										Cells grown aerobically 0.3 (0.3)
	Adaptation time of cells	30 min	1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h	
a_3	...	~50	~50	~20	0.5	0.3	0.5	(<7)	0.35	0.25	
<i>a</i>		~100	<100	30	20	4	6	(15)	3.7	3	5 (15)
<i>c</i>			<100		20	25	6.5	(50)	10	<15	(<100)
c_1			<100		45	25		(70)		30	
b_{560}			200		150		100	90			
b_{556}			No oxidation		500		>200	Reduction

Fig. 6. Cytochrome $a+a_3$ content and ratio cytochrome a_3 /cytochrome *a* of mitochondrial fractions of *S. carlsbergensis* at different stages in adaptation

Cytochrome content was calculated from changes in *E* observed at 445–458 nm in the flow-flash photolysis experiments. Ratios cytochrome a_3 /cytochrome *a* were obtained by measuring changes in *E* in anaerobic photolysis (contribution of cytochrome a_3) and photolysis in the presence of 17 μ M- O_2 (contribution of cytochromes $a+a_3$). Arrows indicate values for mitochondrial fractions from cells grown aerobically to the phase of glucose de-repression.

fraction containing all the detectable *a*- and *c*-type cytochromes. After this time the appearance of these cytochromes in the supernatant fraction obtained after centrifugation at 10⁵g-min indicates either that mitochondrial membranes have become more fragile or that a genuine alteration in subcellular distributions has occurred after what may be regarded as a considerable period of starvation of non-proliferating cells. Thus the validity of regarding spectrophotometric observations on the mitochondrial fraction as being truly representative of these newly functional organelles is acceptable in the earlier stages of adaptation. Interpretation of results obtained with mitochondrial preparations from cells adapted for 7 h or longer must be treated with caution until a further investigation of the basis of altered cytochrome distribution has been made. Increases in *a*-, *b*- and *c*-type cytochromes early in adaptation occur at different rates so as to produce mitochondria with different proportions of cytochromes.

CO-difference spectra reveal the presence of at least one (and possibly two) haemoproteins other than cytochrome a_3 , which contribute to the Soret region of spectra after adaptation for 10 min. One of these, cytochrome *P*-450, cannot be detected after 1 h; this observation agrees with that of Ishidate *et al.*

(1969b) that this haemoprotein disappears during the first 40 min of aeration of anaerobically grown *S. cerevisiae*. The second absorption maximum, which has previously been ascribed to 420-CO pigment (Lindenmayer & Smith, 1964) or cytochrome *P*-420 (Ishidate *et al.*, 1969a; Cartledge & Lloyd, 1972b), may have contributions from one or more of the following haemoproteins containing protohaem in a high spin state: (a) it may arise as a degradation product of cytochrome *P*-450 (Omura & Sato, 1964); (b) it may represent yeast haemoglobin (Keilin, 1953); (c) it might represent absorption due to cytochrome *c* peroxidase (Yonetani & Ray, 1965); (d) it might be due to cytochrome *o*, which has been shown to be present in aerobically grown *S. carlsbergensis* (Mok *et al.*, 1969); (e) it might be due to a cytochrome precursor such as protohaem or the product of cytochrome *b* degradation. Alternative (a) can be excluded as the absorption maximum at 420 nm has been demonstrated in intact cells (Cartledge & Lloyd, 1972b), and the subcellular location in crude mitochondrial fraction would suggest that it is not due to the presence of yeast haemoglobin. The finding that 'cytochrome *P*-420' is increased during adaptation suggests that it does not have a precursor-product relationship with the cytochromes of the mitochondrial electron-transport chain. Spectral data do not reveal the α -band at 590 nm characteristic of cytochrome *c* peroxidase and we have been unable to detect this enzyme by using the assay procedure of Yonetani & Ray (1965).

This CO-reacting haemoprotein should not be termed cytochrome *o* until it has been characterized unequivocally as a functional terminal oxidase at times in adaptation when the cytochrome *a*₃ content is very low or completely absent.

It is evident from the measurement of kinetics of cytochrome oxidation in mitochondria from organisms at different stages of respiratory adaptation that cytochrome-cytochrome interactions occur almost as rapidly in the early stages as in mitochondria that have attained a higher cytochrome content than that found in aerobically grown glucose-depressed cells. This is so irrespective of the finding that the mitochondrial content of *a*-type cytochrome relative to that of cytochromes *b* and *c* is quite variable at different stages. Thus the assembly of functional electron-transport chains must occur as the individual components are synthesized; there is no evidence either for accumulation of extramitochondrial cytochromes as soluble pools, or for extensive cytochrome dislocations in the mitochondria. However, biphasic responses of both cytochromes *c* and *e* were observed on mixing with O₂ in mitochondrial fractions isolated during the early stages of adaptation.

Thanks are due to Dr. E. Keyhani, Dr. T. Ohnishi and Dr. D. Wilson for helpful discussions. This work was carried out during the tenure of a Medical Research Council Post-Graduate Studentship and a Travel Grant from the Wellcome Trust by T. G. C.

References

- Bartley, W. & Tustanoff, E. R. (1966) *Biochem. J.* **99**, 599–603
- Cartledge, T. G. & Lloyd, D. (1972a) *Biochem. J.* **126**, 381–393
- Cartledge, T. G. & Lloyd, D. (1972b) *Biochem. J.* **127**, 693–703
- Chance, B. (1951) *Rev. Sci. Instrum.* **22**, 634–638
- Chance, B. (1953) *J. Biol. Chem.* **202**, 397–406
- Chance, B. (1954) *Discuss. Faraday Soc.* **17**, 120–128
- Chance, B. (1957) *Methods Enzymol.* **4**, 273–329
- Chance, B. (1972) *FEBS Lett.* **23**, 3–20
- Chance, B. & Erecińska, M. (1971) *Arch. Biochem. Biophys.* **143**, 675–687
- Chen, W. C. & Charalampous, F. C. (1969) *J. Biol. Chem.* **244**, 2767–2776
- Chin, C. H. (1952) Ph.D. Thesis, University of Cambridge
- Daniel, R. M. (1970) *Biochim. Biophys. Acta* **96**, 342–345
- Ephrussi, B. & Slonimski, P. P. (1950) *Biochim. Biophys. Acta* **6**, 256–268
- Erecińska, M., Chance, B., Wilson, D. F. & Dutton, P. L. (1972) *Proc. Nat. Acad. Sci. U.S.A.* **69**, 50–54
- Ishidate, K., Kawaguchi, K., Tagawa, K. & Hagihara, B. (1969a) *J. Biochem. (Tokyo)* **65**, 375–383
- Ishidate, K., Kawaguchi, K. & Tagawa, K. (1969b) *J. Biochem. (Tokyo)* **65**, 385–392
- Keilin, D. (1953) *Nature (London)* **172**, 390
- Keilin, D. (1966) in *History of Cell Respiration and Cytochrome* (Keilin, J., ed.), p. 272, Cambridge University Press
- Lindenmayer, A. & Estabrook, R. W. (1958) *Arch. Biochem. Biophys.* **78**, 66–82
- Lindenmayer, A. & Smith, L. (1964) *Biochim. Biophys. Acta* **93**, 445–461
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mok, T. C. K., Rickard, P. A. D. & Moss, F. J. (1969) *Biochim. Biophys. Acta* **172**, 438–449
- Ohnishi, T., Kroger, A., Heldt, H. W., Pfaff, E. & Klingenberg, M. (1967) *Eur. J. Biochem.* **1**, 301–311
- Omura, T. & Sato, R. (1964) *J. Biol. Chem.* **239**, 2370–2379
- Rouslin, W. & Schatz, G. (1969) *Biochem. Biophys. Res. Commun.* **37**, 1002–1007
- Wallace, P. G., Huang, M. & Linnane, A. W. (1968) *J. Cell Biol.* **37**, 207–220
- Wilson, D. F., Koppelman, M. C., Erecińska, M. & Dutton, P. L. (1971) *Biochem. Biophys. Res. Commun.* **44**, 759–766
- Yang, C. C. & Legallais, V. (1954) *Rev. Sci. Instrum.* **25**, 801–807
- Yonetani, T. & Ray, G. S. (1965) *J. Biol. Chem.* **240**, 4503–4508