The Development of Cytochromes during the Cell Cycle of a Glucose-Repressed Fission Yeast, *Schizosaccharomyces pombe* 972h−

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1. Spectrophotometric analysis of intact cells of *Schizosaccharomyces pombe*, harvested from exponentially growing cultures during the phase of glucose repression, revealed the presence of cytochromes $a+a_3$, $c$ and at least two species of cytochrome $b$. 2. An absorption maximum at 554 nm at 77°K, previously attributed to cytochrome $c_1$, has been identified as a $b$-type cytochrome. 3. CO-difference spectra reveal the presence of cytochromes $P-420$ and $P-450$ in addition to cytochrome $a_2$. 4. The cell cycle was analysed by separation of cells into classes representing successive stages in the cell cycle by isopycnic zonal centrifugation. 5. Cytochromes $c_{548}$, $b_{554}$ and $b_{560}$ each exhibited a single broad maximum of synthesis during the cell cycle. 6. Amounts of cytochromes $a+a_3$ and $b_{563}$ (tentatively identified as cytochrome $b_T$ by its reaction on pulsing anaerobic cell suspensions with $O_2$) oscillated in phase, and showed two maxima during the cycle; the second maximum of cytochromes $a+a_3$ was coincident with a maximum of activity of enzymically active cytochrome $c$ oxidase. 7. The amount of cytochrome $P-420$ decreased during the first three-quarters of the cell-cycle, whereas that of cytochrome $P-450$ increased during this period. 8. The discrepancy between spectrophotometric and enzymic assay of cytochrome $c$ oxidase, the changing ratio of cytochrome $a_3$/cytochrome $a$ and the relationship between changes in cellular content of cytochromes and previous observations on respiratory oscillations during the cell cycle are discussed.

In the present study we have characterized the cytochromes in suspensions of intact cells of *S. pombe*, harvested from aerobic exponentially growing cultures during the phase of glucose repression. A quantitative evaluation of changes in cellular content of cytochromes during the cell cycle is presented. The results show that the development of cytochromes $b_{554}$, $b_{560}$ and $c_{548}$ show broad peak patterns, whereas cytochromes $b_{563}$ and $a+a_3$ oscillate in phase together and exhibit two maxima during the cell cycle. Differences in the kinetics of appearance of individual cytochromes in the cycle results in changes in the relative proportions of respiratory-chain components in the mitochondrial membranes.

Materials and Methods

*Maintenance, growth and harvesting of the organism*

*S. pombe* 972h− (kindly supplied by Dr. Urs Leupold, Institute of General Microbiology, University of Bern, Switzerland), was maintained and grown on a defined medium containing 1% (w/v) glucose as described by Poole et al. (1973). Batch cultures (4–6 litres) were grown in a 6-litre capacity
New Brunswick Laboratory Fermentor (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). Forced aeration was at 1 litre of air/min per litre of culture, stirring rate 400 rev./min and the growth temperature was 30°C. The exponentially growing culture was harvested during the phase of glucose repression, when the population reached 2.0 × 10⁷–3.0 × 10⁷ cells/ml, by centrifugation at 2000 rev./min (900 g; rₑᵥ, 18 cm) in the 4 × 1-litre rotor of an International centrifuge at 4°C. All subsequent operations were performed at this temperature.

Cell-cycle analysis

This was done by separation of cells from an exponential culture into size classes representing successive stages in the cell cycle. Isopycnic zonal centrifugation was performed essentially as described by Poole & Lloyd (1973), but with some modifications. Cells (11–16 g wet wt.) were suspended in 10% (w/v) dextran to a total volume of 30 ml and loaded on a linear 27–32% (w/v) dextran gradient (400 ml total vol.) in a Beckman Ti-14 zonal rotor fitted with a B-29 core and liner combination, allowing sample unloading at the rotor edge. Loading was at 3000 rev./min in the Beckman L3-50 centrifuge. After centrifugation at 35000 rev./min for 50 min, the rotor was unloaded at 3000 rev./min and 30 ml/min by using water pumped into the rotor centre. Fractions (20 ml) were collected, diluted to 40 ml with water, and the cells were sedimented quantitatively by centrifugation at 17000 rev./min (37000 g; rₑᵥ, 7.6 cm) for 30 min in the 8 × 50 ml rotor of the Sorvall RB2 centrifuge. Cells were washed once by centrifugation at 6000 rev./min (3300 g; rₑᵥ, 7.6 cm) for 5 min in the same centrifuge and resuspended in 15 ml of a buffer containing 0.25 M-mannitol–50 mM-potassium phosphate (pH 7.4). After retention of 0.1 ml of cell suspension for determination of cell numbers and size distributions, volumes of suspension were taken such that each contained an equal number of cells; cells were sedimented by acceleration to 5000 rev./min followed by immediate deceleration. Equal volumes of the above buffer were used to resuspend each pellet so that the final cell suspensions contained equal cell numbers (0.5 × 10⁶–2.25 × 10⁹ cells/ml).

Analytical methods

Cytochrome spectra. Difference spectra were traced 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 sample-cuvette contents had been sparged with CO for 1 min, after reduction of both cuvette contents by endogenous respiration or by the addition of glucose or Na₂S₂O₄.

The following wavelength pairs and reduced-minus-oxidized extinction coefficients were used: cytochrome a+a₃, 444–458 nm, εₘᵦ 8.5 mm⁻¹·cm⁻¹ (Chance, 1953) and 600–630 nm, εₘᵦ 16 mm⁻¹·cm⁻¹ (Chance & Williams, 1956); cytochrome(s) b, 560–570 nm, εₘᵦ 19 mm⁻¹·cm⁻¹ (Ohnishi et al., 1967); cytochrome c, 548–540 nm, εₘᵦ 18 mm·cm⁻¹ (Ohnishi et al., 1967). Low-temperature intensification factors for absorbance measured at the above wavelength pairs were calculated as the ratio of the observed (path-length corrected) Na₂S₂O₄-reduced minus oxidized difference spectra at 77°K to the absorbance observed in identical experiments at room temperature; these were used to make quantitative the spectra recorded at low temperature (Wilson, 1967). To quantify the CO-difference spectra, the following wavelength pairs and extinction coefficients were used: 453–490 nm, εₘᵦ 91.0 mm⁻¹·cm⁻¹ (cytochrome P-450; Omura & Sato, 1964); 419–432 nm, εₘᵦ 170 mm⁻¹·cm⁻¹ (bacterial cytochrome o; Daniel, 1970); cytochrome a₃, εₘᵦ 91.0 mm⁻¹·cm⁻¹ (Chance, 1957), defined as that component of the cytochrome oxidase complex reacting with CO (Keilin, 1925). Unless otherwise indicated, cells were suspended in 0.25 M-mannitol–50 mM-potassium phosphate buffer, pH 7.4, in all spectrophotometric experiments.

Kinetics of cytochrome oxidation. Measurements of reoxidation rates of cytochromes b and c on mixing anaerobic suspensions of intact cells with 17 μM-O₂ were made as described by Cartledge et al. (1972). Rate constants (K₁ values) for pseudo-first-order reactions and t₁/₂ values were calculated as described by Turner et al. (1971). In some experiments, kinetics of reoxidation of cytochromes were also initiated by the laser-induced dissociation of the reduced cytochrome a₃–CO complex in the presence of O₂ (Chance & Erecinska, 1971).

Determination of size distribution of cells. Measurements of cell length were made microscopically by using an image-splitting eyepiece (Dyson, 1960) and used to calculate cell volumes exactly as described by Poole & Lloyd (1973).

Results

Cytochromes in exponentially growing glucose-repressed cells

When 1% (w/v) glucose is used as reductant, and oxidation of the reference cuvette contents is effected by addition of H₂O₂ to a final concentration of 0.1%, difference spectra at room temperature show absorption maxima at 425 and at about 444 nm, corresponding to the γ bands of b- and a-type cytochromes respectively, 519 nm (β band of cytochrome c) and 550 nm and about 560 nm
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Baseline (oxidized minus oxidized) obtained after aeration of both cuvette contents. Curve (b) was obtained 5 min after addition of glucose (final concn., 1% w/v) to sample cuvette. Curves (a) and (b) were recorded at room temperature with cell suspensions containing 3.5 \times 10^6 cells/ml; the path length was 10 mm and spectral band width 4 nm. In curves (c), (d) and (e), oxidation was by addition of 0.1% \text{H}_2 \text{O}_2 before immersion of cuvettes in liquid N\textsubscript{2} and recording of spectra at 77 K. In curve (c), reduction was achieved by 5 min of incubation with 1% glucose, in curve (d) by addition of glucose to an aerobic cell suspension containing 0.1 \text{mM}-\text{antimycin A}, and in curve (e) by addition of 10\text{mM-ascorbate}+2\text{mM-NNN'N'-tetramethyl-p-phenylenediamine}; cell concentration was 3.0 \times 10^6 cells/ml, path length 2 mm and spectral band width 2 nm throughout.

and at 605 nm, corresponding to the \( \alpha \) bands of cytochromes \( c, b \) and \( \alpha+\alpha_3 \) respectively (Fig. 1b). Similar difference spectra recorded at 77 K (Fig. 1c) revealed a shift in the position of absorption maxima of 2–3 nm to shorter wavelengths.

Additional absorption maxima not clearly defined at room temperature were observed at 524 and 554 nm. Shoulders were evident at 541–542 nm (attributed to the \( \alpha_2 \) band of cytochrome \( c \); Heslot \textit{et al.}, 1970) and 562–564 nm. Addition of dithionite resulted in little apparent change in the spectrum but a shoulder at 580 nm was more apparent in glucose-reduced cells. Addition of glucose to aerobic cell suspensions in the presence of 0.1 \text{mM}-\text{antimycin A} (Fig. 1d) resulted in reduction of \( b \)-type cytochromes, and allowed their contribution (\( \alpha \) bands at 554 and 560 nm, \( \beta \) band at 528 nm, \( \gamma \) band at 430 nm) to be assessed. Reduction with 10 \text{mM-ascorbate}+2 \text{mM-NNN'N'-tetramethyl-p-phenylenediamine} was used to define absorption maxima owing to \( \alpha \)- and \( c \)-type cytochromes and failed to show a maximum at 554 nm, previously attributed to cytochrome \( c_1 \) (Heslot \textit{et al.}, 1970) (Fig. 1e). Absorption maxima at about 438, 580 and 620 nm have not been identified.

The effect of mannitol concentration on the low-temperature intensification at the absorption maxima of the cytochromes (Fig. 2) was determined by dilution of a stock suspension of cells with mannitol solutions in phosphate buffer, yielding final concentrations from 0.05 to 0.72 M-mannitol. The intensification factor for the \( \alpha \) band of cytochromes \( \alpha+\alpha_3 \) increases from 1 to 6.3 as the mannitol concentration is increased from 0.05 to 0.25 M; corresponding values for the Soret band are 1 and 3.25 respectively. Similarly, intensification factors increase from 1.6 to 3.9 and from 1.6 to 4.5 for \( b \)- and \( c \)-type cytochromes respectively over the same range of mannitol concentrations.

CO-difference spectra (endogenous-reduced+CO minus endogenous-reduced; Fig. 3) reveal the presence of CO-reacting haemoprotein(s) other than cytochrome \( a_3 \) (absorption maxima at 453 and 590 nm; minima at 445 and at about 605 nm). The absorption maximum at 453 nm is attributed to cytochrome \( P-450 \). Other absorption maxima are evident at 419, 542–546 and 567 nm and minima at 556 and 575 nm. Addition of glucose to both cuvettes leads to increased absorption at 419 nm and in the \( \alpha \) region of the spectrum, but no further increases in absorption at 435 or 453 nm (Fig. 3c). Subsequent addition of dithionite has similar results. Storage of cell suspensions at 4°C for up to 24 h leads to a progressive decrease in the amounts of CO-reacting haemoproteins reducible by endogenous respiration or by glucose. A decrease in total amounts of cytochrome \( a_3 \) and \( P-453 \) over this period is accompanied by an increase in absorption at 419 nm.

Kinetics of reoxidation of cytochromes

The kinetics of reoxidation of cytochromes were investigated by the regenerative flow technique; typical traces obtained with suspensions of intact cells from an exponentially growing culture are

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Fig. 1. Difference spectra of \( S. \) pombe harvested from exponential cultures during the phase of glucose repression.

(a) Baseline (oxidized minus oxidized) obtained after aeration of both cuvette contents. Curve (b) was obtained 5 min after addition of glucose (final concn., 1% w/v) to sample cuvette. Curves (a) and (b) were recorded at room temperature with cell suspensions containing 3.5 \times 10^6 cells/ml; the path length was 10 mm and spectral band width 4 nm. In curves (c), (d) and (e), oxidation was by addition of 0.1% \text{H}_2 \text{O}_2 before immersion of cuvettes in liquid N\textsubscript{2} and recording of spectra at 77 K. In curve (c), reduction was achieved by 5 min of incubation with 1% glucose, in curve (d) by addition of glucose to an aerobic cell suspension containing 0.1 \text{mM}-\text{antimycin A}, and in curve (e) by addition of 10 \text{mM-ascorbate}+2 \text{mM-NNN'N'-tetramethyl-p-phenylenediamine}; cell concentration was 3.0 \times 10^6 cells/ml, path length 2 mm and spectral band width 2 nm throughout.

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Fig. 2. Effect of mannitol concentration on the low-temperature intensification of cytochrome absorption in suspensions of intact cells of S. pombe

A suspension of cells from an exponentially growing culture was diluted with mannitol solutions in 50mM-potassium phosphate, pH7.4, to give cell suspensions containing 1.21×10⁹ cells/ml in a range of mannitol concentrations (0.05-0.72M). Low-temperature intensification factors for absorbance owing to cytochrome components were calculated as the ratio of absorbance at suitable wavelength pairs in Na₂S₂O₄-reduced-minus-oxidized difference spectra at 77°K to the absorbance observed in a similar spectrum of a cell suspension lacking mannitol at room temperature. Intensification factors for cytochrome c (548-540nm; ○), b-type cytochrome(s) (560-570nm; □), and cytochrome a+a₃ measured at 445-458nm (△) and 600-630nm (▼) are shown. Spectral bandwidth was set at 2nm for spectra at 77°K and 4nm for that at room temperature; path lengths at 77°K and room temperature were 2 and 10mm respectively.

shown in Fig. 4. Anaerobiosis was attained on exhaustion of O₂ by endogenous respiration of the cells. On mixing with 17μM-O₂, oxidation of cytochrome c (observed at 550-540nm) occurred very rapidly at room temperature (K₁ = 157s⁻¹; τ₁ = 15ms; Fig. 4a, trace 3); reoxidation of cytochrome b (observed at 561-540nm) proceeded more slowly (K₁ = 51s⁻¹; τ₁ = 45ms; Fig. 4a, trace 1). Observation at 566-540nm revealed rapid oxidation of another species of cytochrome b (K₁ = 124s⁻¹; τ₁ = 18ms; Fig. 4a, trace 2).

Fig. 3. CO difference spectra of S. pombe harvested from exponential cultures during the phase of glucose repression

Both sample and reference cuvettes contained suspensions of intact cells (1.20×10⁹ cells/ml). (a) Baseline (contents of both cuvettes reduced by endogenous respiration). (b) Difference spectrum obtained at room temperature after contents of sample cuvette had been sparged with CO for 1 min. (c) Spectrum obtained after addition of glucose to sample cuvette to 1% final concn. (d) Spectrum obtained after addition of dithionite to sample cuvettes in (c). Path length throughout was 10mm and spectral band width 2nm.

In a separate experiment, kinetics of reoxidation of b- and c-type cytochromes were initiated by laser-induced dissociation of the reduced cytochrome a₃-CO complex at room temperature in the presence of O₂ and 15.2μM-antimycin A (Fig. 4b). On mixing the anaerobic CO-inhibited cell suspension with 17μM-O₂ and observation at the above wavelength pairs, there was rapid, partial oxidation of species absorbing at all three wavelength pairs. Dissociation of the cytochrome a₃-CO complex by flow-flash photolysis (Chance & Erecińska, 1971) led to rapid oxidation of cytochrome c (τₕ = 17ms; Fig. 4b, trace 3). No oxidation of cytochrome b (observed at 561-540nm; Fig. 4b, trace 1) was evident, owing to the presence of antimycin A, but observation at 566-540nm revealed reduction of a species of cytochrome b (τₕ ~ 100ms; Fig. 4b, trace 2). The immediate reduction of the species of cytochrome b with an absorption maximum at 566nm (cyto-
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Fig. 4. Kinetics of reactions of b- and c-type cytochromes in intact cells of S. pombe, harvested from exponential cultures during the phase of glucose repression

Measurement of kinetics of b- and c-type cytochromes was in the regenerative flow apparatus. (a) Cell suspension (18 ml, containing $10.2 \times 10^6$ cells) was allowed to become anaerobic by endogenous respiration. Oxidation was achieved by mixing the anaerobic suspension with $17 \mu M$-O$_2$; the mixture was driven rapidly through the light-path of the dual-wavelength spectrophotometer and the time-course of oxidation was recorded on a storage oscilloscope. Trace 1, cytochrome b oxidation (561–540 nm); trace 2, cytochrome b oxidation (566–540 nm); trace 3, cytochrome c oxidation (550–540 nm); trace 4, flow velocity trace. (b) Kinetics of reoxidation of respiratory-chain components after photolytic decomposition of CO-liganded cytochrome a$_1$. The CO-inhibited cell suspension (18 ml, containing $10.2 \times 10^6$ cells) was allowed to become anaerobic by endogenous respiration in the presence of 15.2 $\mu M$-antimycin A. Laser-induced dissociation of the cytochrome a$_1$–CO complex 120 ms after mixing with 17 $\mu M$-O$_2$ allows reoxidation of cytochromes. Traces 1–4 are as in (a). All experiments were conducted at 24°C; the path length was 6 mm throughout.

A suspension (30 ml) containing 15.8 g wet wt. of cells was loaded on the dextran gradient in a Beckman Ti-14 (B29) zonal rotor. Centrifugation was at 35000 rev./min for 50 min (2.00 $\times 10^6$ g-min at the sample zone; $\int_0^\infty \omega^2 dt = 4.53 \times 10^{10}$ rad$^2$/s$^2$). Difference spectra of intact cells were recorded in successive fractions removed from the rotor. Fractions 3, 5, 7, 9 corresponded to radial distances from the rotor centre of 5.04, 4.65, 4.40 and 4.02 cm respectively. Contents of sample cuvettes were reduced by 5 min of incubation with 1% glucose. Oxidation of reference suspensions was by addition of 0.1% H$_2$O$_2$; the cuvettes were then immersed in liquid N$_2$ and spectra recorded at 77°C. The path length throughout was 2 mm and spectral band width 2 nm. Cell concentrations were $3.0 \times 10^6$ cells/ml throughout.

Fig. 5. Difference spectra of intact cells after analysis of the cell cycle by isopycnic zonal centrifugation of cells from an exponentially growing culture of S. pombe

Changes in cellular content of cytochromes during the cell cycle

By using isopycnic centrifugation in dextran gradients, cells were separated into classes representing successive stages in the first three-quarters of the

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cell cycle (Poole & Lloyd, 1973). Low-temperature difference spectra (glucose-reduced minus H$_2$O$_2$-oxidized) of intact cells in successive fractions removed from the rotor revealed changes in concentrations of the various cytochrome components during this fraction of the cell cycle (Fig. 5).

Cell volume (Fig. 6a) increases linearly and inversely as a function of distance from the rotor centre. Cytochrome c oxidase has been shown to exhibit a single maximum of activity at 0.67 of a cell cycle (Fig. 6e) in synchronous cultures (Poole & Lloyd, 1973). The timing of this maximum (where mode of the frequency distribution of cell volume is 115 $\mu$m$^3$; Fig. 6e) has been used as a reference point to enable interpretation of the cell cycle across gradients to be made (Fig. 6b).

Absorption owing to $b$-type cytochromes (maxima at 554 and 560 nm; Fig. 6d) increased rapidly over the first one-third of the cell cycle; the amount of the species absorbing at 554 nm further increased until 0.5 of a cell cycle and then decreased, whereas that at 560 nm remained constant during the remaining portion of the cycle studied. A shoulder of absorption owing to cytochrome $b_{563}$ ($b_T$; 563 nm at 77 K; 566 nm at room temperature; Sato et al., 1972) increased more rapidly and at a linear rate for 0.4 of a cell cycle and then decreased (Fig. 6c); a second maximum was evident at 0.67 of a cell cycle. The rate of increase in the cellular content of cytochrome $c$ (548 nm) was rapid during early stages of the cell cycle but became progressively slower at later stages of the cycle (Fig. 6c). Amounts of cytochrome $a+a_3$ measured at both 445–458 nm and 600–630 nm oscillated in phase with the observed fluctuations in content of cytochrome $b_{563}$ ($b_T$; Fig. 6e).

In a similar, separate experiment, contents of CO-reactive haemoproteins, measured in CO-difference spectra (Na$_2$S$_2$O$_4$-reduced + CO minus Na$_2$S$_2$O$_4$-reduced) were measured in cells at successive stages in the cell cycle. There was a progressive decrease in absorption at 419 nm in cells throughout the cell cycle (Fig. 7). This was accompanied by an overall increase in absorption at 453 nm and a

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**Fig. 6.** Cellular contents of cytochromes after analysis of the cell cycle by isopycnic zonal centrifugation of cells from an exponentially growing culture of S. pombe

Conditions of centrifugation were as in Fig. (5). (a) Modes of frequency distributions of cell volumes in successive fractions removed from the rotor. (b) Resolved portion of the cell cycle, represented as a linear scale, and normalized with respect to a cell volume of 115 $\mu$m$^3$ at 0.67 of a cell cycle. (c) Amounts of cytochrome $c_{554}$ (C) and $b_{563}$ (C). (d) Amounts of cytochrome $b_{554}$ (C) and $b_{560}$ (C). (e) Amounts of cytochromes $a+a_3$, measured at 445–458 nm ($\Delta$) and 600–630 nm (×) respectively, together with cytochrome c oxidase activity (-----), plotted as a function of the cell cycle, as determined in a separate similar experiment (Poole & Lloyd, 1973).
more complex pattern of change in absorption at 435 nm owing to cytochrome \( a_3 \). Analysis of these results with respect to the cell cycle (Figs. 8a and 8b) shows that cellular content of cytochrome \( a_3 \) falls to a minimum value at approx. 0.5 of a cell cycle (Fig. 8c). Subtraction of the contribution of cytochrome \( a_3 \) from total content of cytochromes \( a + a_3 \) (Fig. 6d) gave values for cytochrome \( a \) (Fig. 8d) which showed two peaks/cycle in phase with cytochromes \( a + a_3 \) and \( b_T \). The ratio of cytochrome \( a_3/a \) reaches a maximum value of 1.4 at approx. 0.6 of a cell cycle; minimum values for this ratio (about 0.4) were found at 0.4 and 0.7 of the cell cycle (Fig. 8d).

**Discussion**

Reduced-minus-oxidized difference spectra of intact cells of *S. pombe* reveal the presence of \( b- \) and \( a \)-type cytochromes, similar to those observed in other aerobically grown yeasts and those observed in the \( \alpha \) and \( \beta \) regions of absolute absorption spectra of this organism (Heslot *et al.*, 1970). However, results indicate that an absorption maximum at
554 nm is due to a b-type cytochrome and not to cytochrome c₁ as suggested by the above authors. Therefore this organism has three distinct absorption maxima in the α region attributable to b-type cytochromes at 554, 560–561 and 562–564 nm at 77°C. In the present study, the last component (absorption maximum at about 566 nm at room temperature) has been tentatively identified as cytochrome b₃ by its behaviour when anaerobic cell suspensions are pulsed with O₂. An absorption maximum at 554 nm at 77°C has been attributed to one of two α bands of cytochrome b₇ in Candida utilis (Sato et al., 1972). However, the observation that the ratio of absorption at 554 and 562 nm respectively changes during the cell cycle of S. pombe suggests that these maxima are due to distinct species of b-type cytochromes.

The contribution of cytochrome a₃ (435–445 nm in CO-difference spectra) in cells from exponentially growing cultures to the cytochrome a+a₃ complex is 45% (when cytochrome a+a₃ is measured at 600–630 nm in glucose-reduced minus H₂O₂-oxidized difference spectra at 77°C) or 23% when measurement of cytochrome a+a₃ is made at 445–458 nm in the latter spectra. Overestimation of amounts of cytochrome a+a₃ when measured at the latter wavelength pair may result from interference by the trough caused by flavoproteins and non-haem iron at wavelengths close to the reference wavelength (458 nm). The results indicate that the ratio of cytochrome a₃ (measured in CO-difference spectra) to cytochrome a (measured at 600–630 nm in reduced-minus-oxidized difference spectra after correction for the contribution of cytochrome a₃) varies during the cell cycle from 0.34 (at 0.4 of the cell cycle) to 1.4 at 0.6 of the cell cycle, assuming that (1), the dissociation constant of the cytochrome a₃–CO complex does not change during the cell cycle (unlikely since the CO concentration was high compared with the equilibrium constant for CO binding) and (2), the value of ε for the CO–a₃ complex does not alter owing to haem–haem interactions.

Thus the expression of the two spectrophotometrically distinct species of haem a in the cytochrome oxidase complex does not occur simultaneously.

Two alternative hypotheses may be proposed to interpret the observed changes in cellular content of cytochromes a and a₃ through the cell cycle: (1) cytochrome a and cytochrome a₃ are two distinct haemoproteins and their syntheses are under independent control, or (2) cytochrome a is a precursor of cytochrome a₃. The conversion of cytochrome a into cytochrome a₃ may involve (a) a conformational change, or (b) the addition of another component, one or more polypeptides, haem, or Cu. The evidence for both points of view has been presented by Wainio (1970). Interactions between the two haem groups of the cytochrome c oxidase complex indicate that these are in very close proximity and that they may be aligned in such a way that the ligand-binding site (for CO, O₂ etc.) is located between the two haems (Leigh & Wilson, 1972). A previous observation indicates that the synthesis of cytochrome a precedes that of cytochrome a₃, so that the ratio of cytochrome a₃/cytochrome a varies during the process of respiratory adaptation in yeast from 0.6 to 1.15 (Cartledge et al., 1972). Similar results have since confirmed this observation (Chen & Charalampous, 1973).

In the present work, the time-course of development of cytochromes a and a₃ during the part of the cell cycle analysed falls into five phases (Scheme 1). Phases 1 and 4 are equivalent in that over this time the cellular content of cytochrome a rises whereas that of cytochrome a₃ is constant. Phases 3 and 5 are also equivalent in that the content of cytochrome a falls as that of cytochrome a₃ rises. It is during these phases that the extra component(s) (x) may be combined with cytochrome a to give cytochrome a₃, the unknown component(s) being synthesized or made available only at these two periods of the cell cycle. Phase 2 corresponds to destruction of both cytochromes by an unknown mechanism. The time-course of development of cytochrome c oxidase activity parallels the expression of cytochrome a₃.

The observed reciprocal relationship between absorption at 419 nm in CO-difference spectra and the amounts of other haemoproteins on storage of intact cells may indicate that the haemoproteins contributing to this absorption maximum are breakdown products of other cytochrome components. Similar interrelationships between cytochrome P-420 and other cytochromes during the cell cycle may be indicative of a precursor role of components contributing to the absorption maximum at 419 nm. Similar species reacting slowly with CO have been reported in other yeast species (Lindemayer & Smith, 1964; Ishidate et al., 1969; Cartledge et al., 1972), but their precise functions and subcellular localizations have not been elucidated. The presence of cytochrome P-450 in yeasts is usually associated with anaerobic or semi-anaerobic growth (Ishidate et al., 1969; Cartledge et al., 1972) or aerobic growth in the presence of high concentrations (4–10%, w/v) of glucose (Lindemayer & Smith, 1964). The functional significance of this haemoprotein is unclear. In the present system cytochrome P-450 appears to have a physiological function; it is reducible by glucose or endogenous substrates, unlike cytochrome P-420, which is totally reducible only on addition of dithionite.

The validity of representation of the cell cycle across a gradient on a density basis has been confirmed, but the characteristic ‘constant volume’ stage in the last quarter of the cell cycle of S. pombe
(Mitchison, 1957) precludes a complete cycle analysis (Poole & Lloyd, 1973). The requirement that the concentrations of all cellular components double during the cell cycle (Campbell, 1957) enables extrapolation of the results to include changes occurring in the last quarter of the cycle. Thus cytochromes c₅₄₈, b₅₅₄ and b₅₆₁ exhibit broad peak patterns of synthesis during the cell cycle. The ratio and timings of syntheses of the individual cytochromes differ, such that the relative proportions of the components vary widely during the cell cycle. The results clearly demonstrate the flexibility of the composition of the mitochondrial membrane under constant environmental conditions. Changes in the stoichiometry of cytochrome amounts have been reported during respiratory adaptation in yeast (Cartledge et al., 1972) or under differing conditions of glucose repression (Lukins et al., 1968).

Measurements of the shoulders of absorption at 563nm in difference spectra at 77°K owing to cytochrome b₅₆₃ (b₇) suggest that the concentration of this component also oscillates during the cell-cycle in phase with cytochromes a+a₃. The cytochrome b₇ content of Neurospora mitochondria is greatly decreased concomitantly with the amount of cytochrome a+a₃ when hyphae are grown in the presence of chloramphenicol, a specific inhibitor of mitochondrial protein synthesis (von Jagow & Klingenberg, 1972). These and other results (Weiss, 1972) suggest that mitochondrial protein synthesis may contribute to the biosynthesis of certain b-type cytochromes.

The phasing of oscillations in amounts of cytochromes a+a₃ and b₅₆₃ (b₇) in the present study suggests that these two components are subject to a common regulatory mechanism, and unlike other cytochromes their syntheses may require the participation of the mitochondrial protein-synthesizing system.

Previous observations on respiratory oscillations
during the cell cycle suggested involvement of the oscillating component in energy conservation (Poole et al., 1973). The oscillations in amounts of cytochromes $b_\text{r}$ and $a+a_3$ (those components associated with energy coupling at sites II and III of the mitochondrial respiratory chain; Chance, 1972) are not in phase with the observed maxima of respiration rates previously described, nor do they show the same periodicity. We conclude that there is no direct relationship between the observed oscillations of these two cytochromes and the oscillations in overall respiration rates.

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