Oscillations of Enzyme Activities During the Cell-Cycle of a Glucose-Repressed Fission-Yeast *Schizosaccharomyces pombe* 972h⁻

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1. Increased specific activities of cytochrome *c* oxidase, catalase, succinate dehydrogenase, succinate-cytochrome *c* oxidoreductase, NADH-cytochrome *c* oxidoreductase and malate dehydrogenase were observed during glucose de-repression of *Schizosaccharomyces pombe*. 2. The cell-cycle of this organism was analysed by three different methods: (a) harvesting of cells at intervals from a synchronous culture, (b) separation of cells by rate-zonal centrifugation into different size classes and (c) separation of cells by isopycnic-zonal centrifugation into different density classes. 3. Measurement of enzyme activities during the cell-cycle showed that all the enzymes assayed [cytochrome *c* oxidase, catalase, acid *p*-nitrophenylphosphatase, NADH-dehydrogenase, NADH-cytochrome *c* oxidoreductase, NADPH-cytochrome *c* oxidoreductase, succinate dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase (NADP) and fumarate hydratase] show periodic expression as 'peaks'. 4. Cytochrome *c* oxidase shows a single maximum at 0.67 of a cycle, whereas succinate dehydrogenase exhibits two maxima separated by 0.5 of a cell-cycle. 5. All other enzymes assayed showed two distinct maxima per cell-cycle; for catalase, malate dehydrogenase and NADPH-cytochrome *c* oxidoreductase there is the possibility of multiple fluctuations. 6. The single maximum of cytochrome *c* oxidase appears at a similar time in the cycle to one maximum of each of the other enzymes studied, except for NADH dehydrogenase. 7. These results are discussed with reference to previous observations on the expression of enzyme activities during the cell-cycle of yeasts.

The development of methods for producing large-scale synchronously-dividing cultures has enabled the activities of a variety of enzymes to be assayed during the cell-cycle (Mitchison, 1971). It has become clear that the kinetics of enzyme synthesis during the cell-cycle may be divided into two broad classes (Mitchison, 1969): enzyme activities that increase continuously, either at a constant rate or exponentially, as demonstrated by several prokaryotic organisms, and those that increase periodically in a series of 'steps' or 'peaks' as exemplified by most eukaryotic cell types. Possible mechanisms of regulation of these syntheses have recently been reviewed by Halvorson *et al.* (1971).

Attention has largely been focused on those enzymes that are subject to well-documented control mechanisms. There have been relatively few studies on the formation of 'marker' enzymes, associated with specific cell organelles, during the cell cycle. Warmley *et al.* (1970) described linear increases of NADPH-cytochrome *c* oxidoreductase and uridine diphosphatase (microsomal marker enzymes), cytochrome *c* oxidase and succinate-cytochrome *c* oxidoreductase (inner mitochondrial membrane enzymes), and a step-wise increase of glutamate dehydrogenase (mitochondrial matrix enzyme) in the cell-cycle of

neoplastic mast cells. Esterase and uridine diphosphatase (markers for rough and smooth endoplasmic reticulum respectively) and 5'-nucleotidase (plasma membrane enzyme) exhibited one maximum per cycle in synchronized cultures of L51 78Y cells (Bosmann, 1970). Succinate dehydrogenase and monoamine oxidase (mitochondrial enzymes) showed similar patterns in these cells (Bosmann, 1971).

There have been only a few reports of the activities of respiratory enzymes in the cell-cycle of yeast. In *Saccharomyces cerevisiae*, synchronized by a nutritional induction method, both cytochrome *c* oxidase and malate dehydrogenase exhibited a single step-wise increase during the cell-cycle (Cottrell & Avers, 1970). Single steps in the activities of cytochrome *c* oxidase and succinate dehydrogenase were also observed in this organism by Greksák *et al.* (1971). Both groups interpreted these results as evidence for synchronous expression of mitochondrial components. Periodic expression of cytochrome *c* oxidase and succinate-cytochrome *c* oxidoreductase was observed in synchronous cultures of *S. cerevisiae* by Von Meyenburg (1969).

Previous observations of respiratory oscillations in the cell-cycle of the fission-yeast *Schizosaccharo-*

myces pombe (Poole *et al.*, 1973) led us to investigate activities of a number of respiratory enzymes, and also of two other enzymes, acid *p*-nitrophenylphosphatase and catalase, known to be partly associated with membranous structures in other yeasts (Cartledge & Lloyd, 1972). In the present study, measurements of enzyme activities have been made on samples withdrawn at intervals from synchronous cultures, and on cells separated according to their stage in the cell-cycle, by both rate-zonal centrifugation on sucrose gradients (Sebastian *et al.*, 1971) and isopycnic-zonal centrifugation on dextran gradients. The latter method has been used previously for the separation of cells at a particular stage in the cell-cycle to prepare synchronous cultures (Wiemken *et al.*, 1970), but the validity of representation of the cell-cycle across a gradient on a density basis has not previously been confirmed. A preliminary account of part of this work has been presented (Poole & Lloyd, 1972).

Materials and Methods

Organism and growth conditions

S. pombe strain 972h⁻ (kindly supplied by Dr. Urs Leupold, Institute of General Microbiology, University of Bern, Switzerland) was maintained and grown exactly as described previously on a defined medium containing 1% (w/v) glucose (Poole *et al.*, 1973).

Preparation and harvesting of synchronous cultures

Synchronous cultures were prepared by a velocity-sedimentation size-selection method on sucrose gradients in an MSE HS zonal rotor exactly as described previously (Poole *et al.*, 1973). The degree of synchrony was assessed by the method of Blumenthal & Zahler (1962). Cells were harvested at 4°C from culture samples (200ml during the first 2h of synchronous growth, and subsequently 100ml) by acceleration to 5000 rev./min followed by immediate deceleration in the 6×250ml rotor of an MSE18 centrifuge. All subsequent operations were at 4°C. Cells were resuspended quantitatively in 5ml of disruption buffer (50mM-potassium phosphate buffer, pH 7.4) and homogenized (three strokes) in a loose-fitting hand-operated homogenizer (Jencons Scientific Ltd., Hemel Hempstead, Herts., U.K.). A portion (0.5ml) of the suspension was retained for determination of the total cell protein and the remaining volume was used to prepare cell-free extracts.

Fractionation of cell populations by zonal centrifugation

Rate-zonal centrifugation on sucrose gradients. The method was based on the rate-zonal centrifugation method described previously (Poole *et al.*, 1973),

but utilized a shallower sucrose gradient to achieve separation over a greater radial distance in the rotor. An MSE HS zonal rotor was loaded with linear unbuffered aqueous sucrose gradients, 10–40% (w/w), total volume 590ml, as described previously. The exponentially growing culture (5 litres) was harvested by centrifugation for 15 min at 2500 rev./min (1500g; r_{av} , 24cm) in the 6×1 litre rotor of an MSE Mistral centrifuge at 4°C; all subsequent operations were performed at this temperature. The cells were washed once in water and sedimented by acceleration to 5000 rev./min followed by immediate deceleration in the 6×250ml rotor of an MSE18 centrifuge. The cell pellet (14–20g wet wt.) was resuspended to 50ml in 5% (w/w) sucrose and homogenized as described above.

Cell suspension (30ml) was pumped on to the gradient at approx. 15ml/min, followed by an overlay of water (30ml). The rotor was accelerated to 1500 rev./min for 8 min. Unloading was by displacement with 60% (w/w) sucrose at 600 rev./min and 50ml/min and fractions (20–40ml) were collected at 4°C in 50ml centrifuge tubes. Portions (1ml) of each fraction were retained for sucrose determinations, and the remaining volumes of fractions were centrifuged at 9000 rev./min (10000g; r_{av} , 7.6cm) for 10 min in the 8×50ml rotor of an MSE18 centrifuge. Cells were washed once in distilled water by acceleration to 5000 rev./min in the same rotor, followed by immediate deceleration, and resuspended in the disruption buffer (6.5ml). After retention of 1.5ml of cell suspension for determination of total cell protein, cell numbers and cell sizes, the remaining volume was used to prepare a cell-free extract.

Isopycnic-zonal centrifugation on dextran gradients. This was performed essentially as described above for sucrose gradients, but with some modifications. The cells were suspended in 10% (w/v) dextran and loaded on a linear dextran gradient, 27–33% (w/v), 540ml total volume, in the MSE BXIV zonal rotor at 2500 rev./min in an MSE40 centrifuge. After centrifugation at 35000 rev./min for 50 min the rotor was unloaded at 2500 rev./min and 13ml/min with 60% (w/w) sucrose. Longer times of centrifugation produced no change in cell distribution through the gradient. Fractions (20ml) were collected, diluted to 40ml with water, and the cells were sedimented quantitatively by centrifugation at 18000 rev./min (37000g; r_{av} , 7.6cm), for 30 min in the 8×50ml rotor of an MSE18 centrifuge. Cells were treated subsequently exactly as described above for cells separated by rate-zonal centrifugation in sucrose gradients.

Preparation of cell-free extracts

Disruption of organisms was by one slow passage (5ml/min) through a chilled French Press (Milner *et al.*, 1950) at 107 MPa (15 600 lb-in⁻²). The exudate

Table 1. *Enzyme assay methods*For details of *v*, see the text.

Enzyme	Substrate(s)	Other additions	λ (nm)	ϵ (litre $\cdot$ mmol $^{-1} \cdot$ cm $^{-1}$)	Reference	Coefficient of variation, <i>v</i>
Malate dehydrogenase (L-malate-NAD oxidoreductase, EC 1.1.1.37)	100 μ M- <i>cis</i> -Oxaloacetic acid; 23.6 μ M-NADH	36.5 mM-Potassium phosphate buffer, pH 7.2; 3.65 mM-MgCl ₂	340	6.22	Ochoa (1955)	0.14 (5)
Isocitrate dehydrogenase (NADP) (<i>titro</i> -D ₂ -isocitrate-NADP oxidoreductase, carboxylating, EC 1.1.1.42)	165 μ M-DL-Isocitric acid (trisodium salt); 115 μ M-NADP	33 mM-Potassium phosphate buffer, pH 7.0; 3.3 mM-MgCl ₂	340	6.22	Kornberg (1955)	0.20 (7)
Succinate dehydrogenase (succinate-(acceptor) oxidoreductase, EC 1.3.99.1)	6.7 mM-Sodium succinate	96.3 mM-Potassium phosphate buffer, pH 7.4; 3.2 mM-KCN; 0.8 mM 2,6-dichlorophenol-indophenol; 2.18 mM-phenazine methosulphate	600	21	Ells (1959)	0.11 (5)
NAD(PH)-cytochrome <i>c</i> oxidoreductase (EC 1.6.2.1; EC 1.6.2.3)	186 μ M-NADH or 155 μ M-NADPH; 20 μ M-cytochrome <i>c</i>	16 mM-Potassium phosphate buffer, pH 7.2; 10 mM-KCN	550	18.5	Mahler (1955)	0.14 (5); 0.19 (8)
Succinate-cytochrome <i>c</i> oxidoreductase	6.2 mM-Sodium succinate; 20 μ M cytochrome <i>c</i>	16 mM-Potassium phosphate buffer, pH 7.2; 10 mM-KCN	550	18.5		
NADH dehydrogenase (reduced-NAD-(acceptor) oxidoreductase, EC 1.6.99.3)	2.3 μ M-NADH	36.5 mM-Potassium phosphate buffer, pH 7.2; 3.65 mM-MgCl ₂	340	6.22	Ochoa (1955)	0.16 (5)
Cytochrome <i>c</i> oxidase (ferro-cytochrome <i>c</i> -oxygen oxidoreductase, EC 1.9.3.1)	8 μ M-Cytochrome <i>c</i> , previously reduced with sodium borohydride	18 mM-Potassium phosphate buffer, pH 7.4	550	18.5	Smith (1955)	0.13 (5)
Catalase (H ₂ O ₂ -H ₂ O ₂ oxidoreductase, EC 1.11.1.6)	14 mM-H ₂ O ₂	20 mM-Potassium phosphate buffer, pH 7.2	240	43.6	Lück (1963)	0.11 (7)
Acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2)	5.33 mM- <i>p</i> -Nitrophenyl phosphate	40 mM-Sodium acetate buffer, pH 4.2; 0.02% (w/v) Triton X-100	420	16	Torriani (1960)	0.11 (5)
Fumarate hydratase (L-malate hydro-lyase, EC 4.2.1.2)	28 mM-L-Malic acid	33.3 mM-Potassium phosphate buffer, pH 7.4	240	2.11	Racker (1950)	0.20 (7)

was centrifuged at 4000 rev./min (2000g; r_{av} . 7.6 cm) for 5 min in the 16 × 15 ml rotor of an MSE 18 centrifuge to remove unbroken cells and cell-wall fragments, and the supernatant (termed the cell-free extract) was decanted, care being taken to avoid disturbance of the lower layers.

Enzyme assays

Details of enzyme assays are presented in Table 1. Spectrophotometric assays were performed at room temperature ($18 \pm 2^\circ\text{C}$), with a Cary model 14 recording spectrophotometer, fitted with a slide wire with a full-scale deflexion corresponding to an extinction change of 0.1. Acid *p*-nitrophenylphosphatase was assayed at 30°C . A suitable dilution of cell-free extract was used in the assays to ensure that reaction rates were proportional to enzyme concentrations. Values presented are the means of two determinations at different protein concentrations. All assays were performed within 12 h of preparation of the extracts. Enzyme units are expressed as nmol of substrate transformed/min or as nmol of measured product formed/min.

Determinations of units of enzyme activities were performed on extracts prepared from cell suspensions over a range of cell concentrations between 0.9×10^7 and 115×10^7 cells/ml, and the results were used to calculate the degree of precision of the enzyme assay methods used. Results from these trials, expressed as enzyme units/ 10^7 cells, were used to calculate standard deviations and subsequently the coefficient of variation v , for each assay, where $v = (\text{standard deviation})/(\text{mean})$. In those experiments where units of activity are corrected for cell numbers (i.e. those in which cell populations are separated through gradients), the calculated values of v are expected to represent the maximum error inherent in determination of enzyme activity.

Other determinations

Protein. Protein was measured in cell-free extracts by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. Total cell protein was determined in 0.5 M-NaOH extracts as described by Herbert *et al.* (1971) with the same standard.

Glucose and ethanol. These were estimated by using Sigma reagent kits, numbers 510 and 330-UV respectively.

Size distribution of cells. Measurements of cell-size were made by phase-contrast microscopy, with an image-splitting eyepiece (Dyson, 1960), calibrated with a stage micrometer slide (Graticules Ltd., Tonbridge, Kent, U.K.). At least 100 randomly selected cells were measured in each selected fraction, according to the criteria of Mitchison (1970). The cell diameter, which is constant through the cell-cycle

(Mitchison, 1957), was $3.72 \mu\text{m} \pm 0.21 \mu\text{m}$ (100). The cell volume (Mitchison, 1957) was calculated from the formula for a cylinder with hemispherical ends, $V = [D^2(L - D/3)]/4$, where V = volume (μm^3), L = length (μm) and D = diameter (μm).

Density determinations. Sucrose concentrations were measured by using a refractometer, and densities were calculated by using the results of de Duve *et al.* (1959). Dextran densities were measured by reference to a calibration curve relating refractometer readings to directly measured densities.

Chemicals

Cytochrome *c* (type III, horse heart), NADH (type III, yeast), NADPH (type I), *p*-nitrophenyl phosphate, NADP (yeast), DL-isocitric acid (trisodium salt, type I), phenazine methosulphate, *cis*-oxaloacetic acid (Grade I) and dextran (chemical grade, average mol.wt. 40000) were all from Sigma (London) Chemical Co., London S.W.6, U.K. Bovine serum albumin was from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. The sucrose used was mineral water sugar from Tate and Lyle Ltd., Cardiff, U.K.

Results

Changes in enzyme activities during growth of an asynchronously dividing culture

Exponential growth occurred (mean generation time 2.7 h) until the glucose concentration fell to 10 mM (Fig. 1a). In this phase of growth there was a rapid accumulation of ethanol, but the cells were still glucose repressed as shown by the low activities of all enzymes assayed (Figs. 1b–d). Glucose repression has been demonstrated in this strain of *S. pombe* by Heslot *et al.* (1970). All enzymes assayed, with the exception of acid *p*-nitrophenylphosphatase, showed increased specific activities after growth for 16 h. In all the experiments subsequently described, cells were harvested when cell numbers were between 2.2×10^7 and 2.7×10^7 organisms/ml; at this stage they were still glucose repressed.

Changes in enzyme amounts during the growth of synchronously dividing cultures

Cell numbers increased in a stepwise manner through two doublings and gave satisfactory synchrony indices (Poole *et al.*, 1973) for each cell division (Fig. 2a). Assays of total protein showed that this increased exponentially in the culture (Fig. 2a), whereas the assays of released protein in the cell-free extracts indicated that there was no marked variation in the susceptibility of organisms to disruption at different times in the cell-cycle. Between 40 and 60%

Fig. 1. Changes in cell population, glucose and ethanol concentrations in the medium, and specific activities of enzymes in cell-free extracts from *S. pombe* during growth

(a) shows the cell concentration (cells/ml, ●), glucose concentration (□) and ethanol concentration (■). (b) shows activities of cytochrome c oxidase (●), catalase (○) and acid p-nitrophenylphosphatase (● --- ●). (c) shows activities of succinate dehydrogenase (▽) and succinate-cytochrome c oxidoreductase (□). (d) shows activities of NADH-cytochrome c oxidoreductase (■) and malate dehydrogenase (●). The enzyme activities are all expressed as specific activities (nmol/min per mg of protein).

of the total cell protein was recovered in the extracts, and the measured efficiencies of cell breakage for each sample were used in the calculation of total

enzyme units in the culture. Total amounts of cytochrome c oxidase increased exponentially overall, but rose to a maximum during the middle half of the

Fig. 2. Changes in cell population, total cell protein, protein in cell-free extracts and amounts of enzymes during growth of a synchronously dividing culture of *S. pombe*

(a) shows the synchrony indices (F_1 and F_2) of the first and second doublings in cell numbers respectively (●), and total (□) and released (○) protein of cell suspensions used to prepare cell-free extracts. Vertical lines indicate the midpoints of doublings in cell numbers. (b) shows amounts of cytochrome *c* oxidase (●), catalase (○), acid *p*-nitrophenylphosphatase (●—●), NADH-cytochrome *c* oxidoreductase (■) and succinate dehydrogenase (▽). Amounts of enzymes are expressed as units/ml of culture.

cell-cycle and fell during cell division (Fig. 2b). Catalase and *p*-nitrophenylphosphatase also exhibited exponential increases (Fig. 2b), but the patterns of variation were more complex. Succinate dehydrogenase and NADH-cytochrome *c* oxidoreductase (Fig. 2b) both showed overall exponential increases in amounts, rising to maxima twice per cell-cycle.

Cell-cycle analysis by zonal centrifugation

Two methods of analysis of exponentially-growing populations into classes of cells that represent successive stages in the cell-cycle were used. In the first of these, cells were separated on a size basis by rate-zonal centrifugation through a sucrose gradient. Fig. 3(a) shows variation in cell volumes in individual

Fig. 3. Size distributions of cell populations in fractions after zonal centrifugation of an exponentially growing culture of *S. pombe*

(a) shows the size distributions of cells in five fractions after rate-zonal centrifugation of cells from an exponentially growing culture. A suspension (30ml) containing 6.7g wet wt. of cells was loaded on the gradient in an MSE HS zonal rotor. Centrifugation was at 1500rev./min for 8min; ($1.15 \times 10^4 \text{ g} \cdot \text{min}$ at the sample zone; $\int_0^t \omega^2 \cdot dt = 1.58 \times 10^4 \text{ rad}^2 \cdot \text{s}^{-1}$). (b) shows the size distributions of cells in six fractions after isopycnic zonal centrifugation of cells from an exponentially-growing culture. A suspension (30ml) containing 10.6g wet wt. of cells was loaded on the gradient in a BXIV zonal rotor. Centrifugation was at 35000rev./min for 50min; ($2.34 \times 10^6 \text{ g} \cdot \text{min}$ at the sample zone; $\int_0^t \omega^2 \cdot dt = 4.88 \times 10^{10} \text{ rad}^2 \cdot \text{s}^{-1}$). In both (a) and (b) the broken lines show the size distribution of cells in an exponentially growing culture.

fractions obtained and compares these with the volume distribution of cells from the exponential culture. The modes of the frequency distributions of cell volumes increased almost linearly with distance from the rotor centre. Because cell volume increases linearly during the first three-quarters of the cell-cycle in this organism (Mitchison, 1957), this fraction of the cell-cycle was represented as a linear function of rotor radius. In the second method, cells were separated on a density basis by isopycnic-zonal centrifugation in dextran gradients. Fig. 3(b) shows the variation in cell volumes in individual fractions obtained, and again compares these with the volume distribution of cells from the exponential culture. The observed reciprocal linear relationship between mean cell volume and density confirms the calculations of Mitchison (1957). In this case the first three-quarters of the cell-cycle was inversely related to distance from the rotor centre. Bimodal distributions

of cell volume in fractions representing the last quarter of the cell-cycle were obtained in both methods; neither rate nor isopycnic centrifugation enable the last quarter of the cell-cycle to be resolved.

Analysis by rate-zonal centrifugation. The distribution of cell numbers, mean cell length and modes of frequency distributions of cell volumes throughout the fractions, after centrifugation of cells from an exponentially-growing culture through a shallow sucrose gradient, are shown in Fig. 4(a). Of the enzymes assayed, cytochrome *c* oxidase (Fig. 4b) showed the least variation in amount per cell in the fractions studied; a single trough and peak is evident. Two maxima were seen for all the other enzymes assayed, acid *p*-nitrophenylphosphatase and catalase (Fig. 4b), NADH-cytochrome *c* oxidoreductase and NADH dehydrogenase (Fig. 4c) and malate dehydrogenase and succinate dehydrogenase (Fig. 4d). There

Fig. 4. Analysis of the cell cycle by rate-zonal centrifugation of cells from an exponentially growing culture of *S. pombe*

A suspension (30 ml) containing 6.7 g wet wt. of cells was loaded on the gradient in an MSE HS zonal rotor. Centrifugation was at 1500 rev./min for 8 min; ($1.15 \times 10^4 g \cdot \text{min}$ at the sample zone; $\int_0^t \omega^2 \cdot dr = 1.58 \times 10^4 \text{ rad}^2 \cdot \text{s}^{-1}$). (a) shows distribution of cell numbers (●), mean cell length (□), modes of frequency distributions of cell volumes (■) and sucrose-density gradient (○). (b) shows amounts of cytochrome *c* oxidase (●), catalase (○) and acid *p*-nitrophenylphosphatase (●---●). (c) shows amounts of NADH-cytochrome *c* oxidoreductase (■) and NADH dehydrogenase (▼). (d) shows amounts of succinate dehydrogenase (▽) and malate dehydrogenase (●). Amounts of enzymes are expressed as units/ 10^7 cells.

Fig. 5. Analysis of the cell-cycle by isopycnic-zonal centrifugation of cells from an exponentially growing culture of *S. pombe*

A suspension (30 ml) containing 8.4 g wet wt. of cells was loaded on the gradient in a BXIV zonal rotor. Centrifugation was at 35 000 rev./min for 50 min; ($2.34 \times 10^6 g \cdot \text{min}$ at the sample zone; $\int_0^t \omega^2 \cdot dt = 4.88 \times 10^{10} \text{ rad}^2 \cdot \text{s}^{-1}$). (a) shows distribution of cell numbers (●), mean cell length (□), mode of frequency distributions of cell volumes (■) and dextran-density gradient (○). (b) shows amounts of cytochrome *c* oxidase (●), catalase (○) and acid *p*-nitrophenylphosphatase (●---●). (c) shows amounts of NADH-cytochrome *c* oxidoreductase (■) and NADH dehydrogenase (▼). (d) shows amounts of succinate dehydrogenase (▽) and malate dehydrogenase (●). Amounts of enzymes are expressed as units/ 10^7 cells.

Fig. 6. Analysis of the cell-cycle by isopycnic-zonal centrifugation of cells from an exponentially growing culture of *S. pombe*

A suspension (30 ml) containing 10.6 g wet wt. of cells was loaded on the gradient in a BXIV zonal rotor. Centrifugation was at 35000 rev./min for 50 min; (2.34×10^6 g·min at the sample zone; $\int_0^t \omega^2 \cdot dt = 4.88 \times 10^{10} \text{ rad}^2 \cdot \text{s}^{-1}$). (a) shows distribution of cell numbers (●), mean cell length (□), mode of frequency distributions of cell volumes (■) and dextran-density gradient (○). (b) shows amounts of cytochrome *c* oxidase (●) and isocitrate dehydrogenase (NADP) (□). (c) shows amounts of NADPH-cytochrome *c* oxidoreductase (▲) and fumarate hydratase (○). Amounts of enzymes are expressed as units/ 10^7 cells.

is a possibility of a third maximum at cell division for catalase and malate dehydrogenase.

Analysis by isopycnic-zonal centrifugation. The distribution of cell numbers, mean cell length and modes of frequency distributions of cell volumes throughout the fractions after centrifugation of cells from an exponentially-growing culture to their equilibrium densities in a dextran gradient are shown in Fig. 5(a). For cytochrome *c* oxidase a single minimum and maximum was again observed (Fig. 5b). Two maxima were again evident for all other enzymes assayed (Fig. 5b–d), and again there is the possibility of third maxima for catalase (Fig. 5b) and malate dehydrogenase (Fig. 5d). In a similar experiment (Fig. 6a) the single maximum of cytochrome *c* oxidase occurred at a similar density to that observed in the previous experiment. Over the part of the cell-cycle represented, two maxima of isocitrate dehydro-

genase (NADP) (Fig. 6b) and fumarate hydratase (Fig. 6c) were evident whereas there is the possibility of three maxima of NADPH-cytochrome *c* oxidoreductase (Fig. 6c).

Discussion

All the enzymes investigated show periodic patterns of expression as 'peaks' rather than 'steps' during their overall doubling in the cell cycle of *S. pombe*. In all cases the observed fluctuations in enzyme amounts are much greater than the calculated coefficients of variation, which are a measure of experimental precision. The results obtained from three independent methods of analysis of the cell-cycle may be conveniently compared by means of cell-cycle maps (Scheme 1), which indicate the relative timings of maxima of enzyme amounts during the

Scheme 1. *Timings of maxima of enzyme amounts during the cell-cycle of S. pombe*

In (a) the abscissa represents (as a linear scale extending from 0–1.0) the cell-cycle taken as the period between the mid-points of the first and second doublings in cell numbers in synchronous cultures. (b) shows the results of analysis of the cell-cycle by rate-zonal centrifugation, assuming the cell-cycle to be represented linearly with distance from the rotor centre, and by using the single maximum of cytochrome *c* oxidase at 0.67 of a cell-cycle as a reference point for timings of maxima of other enzymes. (c) shows the results of analyses of the cell-cycle by isopycnic-zonal centrifugations, assuming the cell-cycle to be represented linearly as a reciprocal function of distance from the rotor centre and again normalizing the positions of maxima of enzyme amounts with respect to the position of the maximum of cytochrome *c* oxidase. ▼ indicate the positions of maxima of enzyme amounts; ▽ indicate the possible occurrence of maxima. Arrows with cross bars are mean values with s.d. of timings of maxima determined from results obtained with synchronously dividing cultures.

interval between successive doublings in cell numbers. Analysis of cells harvested at different stages of growth of a synchronous culture has the advantages that the entire cell-cycle may be studied, and that it is possible to confirm the results obtained in successive cycles. On the other hand, the quantity of cells that can be obtained is limited, and time-resolution for events occurring in organisms with short cell-cycles may present technical difficulties. Representation of the cell-cycle across a gradient by either of the two methods described eliminates the deficiencies inherent in the analysis of synchronous cultures, but the characteristic constant-volume stage in the cell-cycle of *S. pombe* (Mitchison, 1957) precludes a complete analysis, as the last quarter of the cell-cycle cannot be resolved.

The single maximum of cytochrome *c* oxidase occurs at 0.67 of the cell-cycle, as determined in extracts of cells from synchronous cultures (Scheme 1). The position of this maximum has been used as a reference point to enable interpretation of the cell-cycle across gradients to be made. Succinate dehydrogenase exhibits two maxima separated by 0.5 of the cell-cycle. The two maxima of NADH-cytochrome *c* oxidoreductase and the two clearly resolved maxima of malate dehydrogenase occur at similar times in the cell-cycle. These patterns of enzyme expression as 'peaks' contrast with the 'step' patterns previously reported for cytochrome *c* oxidase and malate dehydrogenase, which both showed single abrupt stepwise increases during the cell-cycle of galactose-grown *S. cerevisiae* (Cottrell & Avers, 1970). Greksák *et al.* (1971) have also reported single-step increases for both cytochrome *c* oxidase and succinate dehydrogenase in synchronous cultures of *S. cerevisiae*.

Catalase activity showed relatively little variation through the cell-cycle and it is difficult to distinguish between the possible occurrence of two or three maxima. Similar changes in catalase activity have been observed in glucose-grown *S. cerevisiae* (Gifford & Pritchard, 1969). The two maxima observed for acid *p*-nitrophenylphosphatase contrast with the continuous synthesis of this enzyme previously demonstrated in the cell-cycle of *S. pombe* strain 132 grown under similar conditions (Mitchison & Creanor, 1969).

The single maximum of cytochrome *c* oxidase appears at a similar time in the cell-cycle to one maximum of each of the other enzymes assayed with the possible exception of NADH dehydrogenase. The maxima observed in the first half of the cell-cycle for the different enzymes vary widely in their timings.

It has been suggested (Poole *et al.*, 1973) that the oscillations in O₂ uptake which occur with a periodicity of 0.5 of a cell-cycle in this organism may be controlled by the periodic expression of a rate-limiting entity. Although succinate dehydrogenase shows a similar periodicity, the oscillations in amount

of this enzyme are not in phase with those of O₂ uptake. Cytochrome *c* oxidase shows a periodicity unique amongst the respiratory enzymes studied. It appears that a factor other than any of the expressed enzyme activities assayed controls the overall respiratory activity of the organism.

If enzyme amounts reflect concentrations of enzyme proteins then the observation that different enzymes of a particular membrane system (e.g. cytochrome *c* oxidase and succinate dehydrogenase of the inner mitochondrial membrane) are not expressed at identical times in the cell-cycle suggests that the stoichiometry of individual enzyme components in a membrane may vary at different stages in the cell-cycle.

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