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Mitochondrial Adenosine Triphosphatase of the Fission Yeast, *Schizosaccharomyces pombe* 972h⁻

CHANGES IN ACTIVITY AND OLIGOMYCIN-SENSITIVITY DURING THE CELL CYCLE OF CATABOLITE-REPRESSED AND -DE-REPRESSED CELLS

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1. Changes in activity of ATPase (adenosine triphosphatase) during the cell cycle of *Schizosaccharomyces pombe* were analysed in cell-free extracts of cells harvested from different stages of growth of synchronous cultures and also after cell-cycle fractionation. 2. Oligomycin-sensitive ATPase oscillates in both glucose-repressed and glucose-de-repressed synchronous cultures and shows four maxima of activity approximately equally spaced through the cell cycle. The amplitude of the oscillations accounts for between 13 and 80% of the total activity at different times in the cell cycle. 3. Oligomycin sensitivity varies over a fourfold range at different stages of the cell cycle. 4. The periodicity of maximum oligomycin sensitivity is one-quarter of a cell cycle. 5. These results were confirmed for the first three-quarters of the cell cycle by cell-cycle fractionation. 6. In cells growing synchronously with glycerol, ATPase activity increases in a stepwise pattern, with two steps per cell cycle; the first of these occurs at 0.54 of the cell cycle and the second at 0.95. 7. These results are discussed in relation to previously obtained data on the development of mitochondrial activities during the cell cycle.

Previous work has shown that the development of respiratory activity during the cell cycle of the fission yeast *Schizosaccharomyces pombe* occurs discontinuously. In cells growing synchronously in the presence of 1% glucose, many of the enzymes of terminal respiration are catabolite-repressed, and respiration rates and enzymes exhibit peak patterns of expression, as do cytochromes $b_{563}(b_T)$ and $a+a_3$ (Poole *et al.*, 1973; Poole & Lloyd, 1973; Poole *et al.*, 1974). Cells growing synchronously on glycerol also show periodic expression of respiratory activities, but, under these catabolite-de-repressed conditions, rates of respiration, cytochromes and cytochrome *c* oxidase activity all show stepwise expression, whereas the activities of other enzymes assayed again show two distinct maxima during the cell cycle (Poole & Lloyd, 1974).

The mitochondrial ATP synthetase complex of *S. pombe* has been studied by Goffeau *et al.* (1972, 1973). The F_1-ATPase* from glycerol-grown *S. pombe* is similar to the mammalian enzyme and to that from *Saccharomyces cerevisiae* (Kagawa & Racker, 1966; Schatz *et al.*, 1967; Tzagoloff & Meagher, 1971). The biosynthesis of the ATP synthetase complex in yeast (like that of cytochrome *c* oxidase) is under the dual control of the mitochondrial and extramitochondrial protein-synthesis systems; polypeptide subunits of both F_1-ATPase and the oligomycin-sensitivity-conferring peptide are coded for by nuclear DNA and synthesized on cytoplasmic ribosomes, whereas the four polypeptides of the membrane factor (F_0) are synthesized on mitochondrial ribosomes (Tzagoloff, 1969, 1970, 1971; Tzagoloff *et al.*, 1972, 1973; Landry & Goffeau, 1972).

In the present study we have used synchronous cultures and the technique of cell-cycle fractionation to study the time-course of development of ATPase during the cell cycle of *S. pombe*, under both catabolite-repressed and -de-repressed conditions. As with cytochrome *c* oxidase, ATPase activity is expressed periodically, as peaks in glucose-grown cultures, and as steps in glycerol-grown cultures. Sensitivity to oligomycin shows marked variations during the cell cycle. This suggests that analysis of events in synchronous cultures provides a powerful approach to the elucidation of the temporal sequence and mechanism of assembly of subunit polypeptides into functional ATP synthetase under conditions of normal balanced growth.

Experimental

Maintenance, growth and harvesting of the organism

S. pombe 972h⁻ was maintained on 3% (w/v) malt extract/agar and grown with forced aeration in

* Abbreviation: ATPase, adenosine triphosphatase.

minimal media containing either 1% glucose or 1% glycerol, exactly as described previously (Poole *et al.*, 1973; Poole & Lloyd, 1974).

Preparation and harvesting of synchronous cultures

Synchronous cultures were prepared by the continuous-flow size-selection procedure described by Lloyd *et al.* (1975). Cultures were grown until the population of organisms was approx. 4×10^7 cells/ml; at this stage, in growth with glucose, they were catabolite-de-repressed (Poole & Lloyd, 1974). The culture was then delivered with a flow rate of 1 litre/min into the continuous-flow rotor of an MSE 18 centrifuge operating at 3000 rev./min. Under these conditions rate-separation of organisms occurred so as to leave about 10% of the original cell population in the rotor effluent. This effluent, containing the smallest cells of the culture, was force-aerated at a flow rate of 1 litre of air/litre of culture. Zero time for the growth of this synchronous culture was taken when half the culture had passed through the continuous-flow rotor. The degree of synchrony was assessed by the synchrony index (F) of Blumenthal & Zahler (1962), which has a value of 1.0 for a theoretically perfect synchronous culture, and 0 for an exponential culture. Organisms were counted in a Thoma haemocytometer slide (Hawkesley, Lancing, Sussex, U.K.) or in a Coulter Counter (model Z_B; Coulter Electronics Ltd., High St. South, Dunstable, Beds., U.K.). Measurements of cell size were also performed with a Coulter Counter fitted with a P64 Channelyzer (Coulter Electronics Ltd.). Harvesting of synchronous cultures and washing of cells was as described for asynchronous cultures (Lloyd & Edwards, 1976).

Cell-cycle fractionation by zonal centrifugation

This was by rate-zonal centrifugation on a linear gradient in an HSA zonal rotor running in an MSE18 centrifuge as described previously (Poole & Lloyd, 1973). Sedimentation of the organisms was at 1500 rev./min for 5 min.

Preparation of cell-free extracts

Disruption of organisms in 50 mM-Tris/H_2SO_4 buffer, pH 8.6, was by one slow passage through a chilled French Press (Milner *et al.*, 1950) at 107 MPa (15600 lb/in^2) or by focused-field sonication for 30 s at 1 MHz (Coakley, 1971). The resulting suspension was centrifuged at 4000 rev./min (2000*g*; $r_{av.}$ 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; the supernatant (termed the cell-free extract) was decanted, care being taken to avoid disturbance of the lower layers. Assays were performed on crude extracts rather than on purified submitochondrial particles to account for pools of unincorporated precursors of mitochondrial ATPase; under the assay conditions used, the contribution of ATPases of membranes other than mitochondria was minimal.

ATPase assays

ATPase (EC 3.6.1.3) was assayed by both proton release and P_i release as described previously (Lloyd & Edwards, 1976).

Other determinations

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

Glucose. Glucose was determined by using Sigma kit 510.

Sucrose. Sucrose concentrations were measured by using the results of de Duve *et al.* (1959).

Chemicals

ATP, carbonic anhydrase and oligomycin were all from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. The oligomycin is a 17:3 (w/w) mixture of oligomycin B and oligomycin A. 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, Wales, U.K.

Results

Changes in ATPase activity and oligomycin-sensitivity during synchronous growth in the presence of glucose

Continuous-flow size selection gave 10% of the total population of a glucose-grown culture. These small cells were collected from the rotor effluent in a second continuous-flow centrifuge (Type Lab 102B-25; Alfa Laval Co., Great West Rd., Brentford, Middx., U.K.) and were finally resuspended in fresh growth medium containing 1% glucose. Forced aeration of this suspension gave a synchronous culture growing under conditions of catabolite repression. Cell numbers increased in a stepwise manner through two doublings (Fig. 1*a*), and the degree of synchrony was satisfactory as indicated by synchrony indices. The duration of the second cell cycle (i.e. the time-interval between the first and second doubling in cell numbers; 2.8 h) was similar to the mean generation time of the exponential culture from which the cells had been selected. The first cell cycle was always shorter than the second cycle in this type of experiment; this was a consequence of delays incurred during harvesting and resuspension of the cells. The glucose concentration in the medium decreased from 60 to 45 mM during the experiment (Fig. 1*a*). Total protein in the culture showed an exponential increase (Fig. 1*a*). The protein

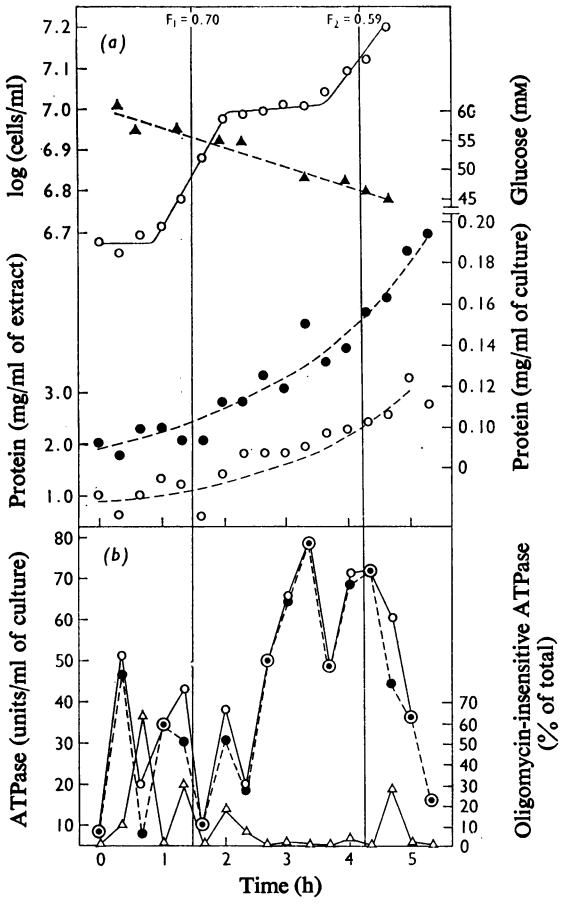


Fig. 1. *Changes in cell population, total cell protein, protein in cell-free extracts, ATPase activities and oligomycin sensitivity during synchronous growth of S. pombe in the presence of* 1% *glucose*

An exponentially growing culture containing 4.9×10^7 cells/ml was subjected to continuous-flow size selection. Harvesting of the smallest cells (10% of total population) by passage through a second continuous-flow centrifuge was followed by resuspension in fresh growth medium containing 1% glucose. (*a*) Synchrony indices (F_1 and F_2) for the first and second doublings in cell numbers (○), glucose concentration in the medium (▲), total protein in the culture (●) and protein concentration of the extracts (○). Vertical lines indicate midpoints in doublings of cell numbers. (*b*) Total ATPase at pH8.6 (○), oligomycin-sensitive ATPase (●) and oligomycin-insensitive ATPase (△, expressed as % of total activity). ATPase activity is expressed as enzyme units/ml of culture.

concentration of cell-free extracts also increased exponentially; thus there was no marked variation in the susceptibility of organisms to breakage at different times in the cell cycle. Efficiencies of disruption were calculated from these protein measurements, and the values obtained were used in each calculation of total enzyme units in the culture.

In the complete cell cycle, between the first and second doublings in cell numbers, three distinct maxima of ATPase activity are evident (Fig. 1*b*); the amplitude of the oscillation corresponds to about 70% of the total activity. More than 70% of the enzyme activity was sensitive to oligomycin; however, the proportions of oligomycin-sensitive and oligomycin-insensitive ATPase varied throughout the experiment (Fig. 1*b*).

Continuous-flow size selection of 16% of the total population of a catabolite-de-repressed culture (glucose concentration 16mM) was used to establish the synchronous culture shown in Fig. 2. The glucose concentration in the medium decreased from 16mM to 5mM during two doublings in cell numbers (Fig. 2*a*). ATPase activity shows a complex pattern of expression (Fig. 2*b*): both assay methods indicated the presence of two major maxima and the possible occurrence of two other maxima between these. The amplitude of the oscillation corresponds to between 40 and 70% of the total activity. At the start of the second cycle ATPase activity was higher at pH6.8 than that at pH8.6, whereas over the last two-thirds of both cell cycles enzyme activity was higher at the higher pH value. However, a close correspondence between the profiles of enzyme activity at the two pH values was evident throughout, and the ratio of activities at these pH values did not vary greatly.

Sensitivity of ATPase to inhibition by oligomycin varies in cell-free extracts prepared at different times in the cell cycle (Fig. 3); these inhibitor titrations enabled the evaluation of I_{50} values and assessment of oligomycin-sensitive and oligomycin-insensitive ATPases (Fig. 2*d*). Values for I_{50} for oligomycin showed a striking (4.5-fold) variation through the cell cycle (Fig. 2*c*); the period of this oscillating inhibitor response was approx. 0.25 of a cell cycle. The activity of oligomycin-sensitive ATPase showed four maxima during the cell cycle, whereas the oligomycin-insensitive enzyme showed only two maxima of activity during the same time-interval.

Cell-cycle analysis of glucose-de-repressed organisms; changes in oligomycin-sensitivity of ATPase

Rate-zonal centrifugation through a sucrose gradient separates classes of cells that represent successive stages in the cell cycle (Poole & Lloyd, 1973). Cell volume increases linearly during the first three-quarters of the cell cycle and then remains constant while a cell plate is formed (Mitchison, 1971); the last quarter of the cycle cannot therefore be investigated by this procedure.

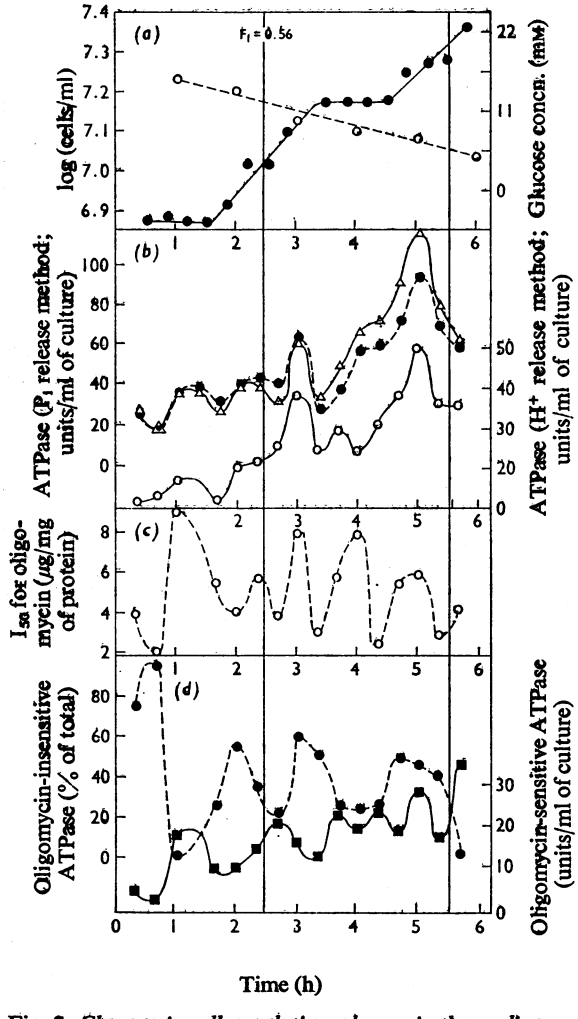


Fig. 2. *Changes in cell population, glucose in the medium, ATPase activities and oligomycin sensitivity during synchronous growth of glucose-de-repressed S. pombe*
Continuous-flow size-selection synchrony was established by allowing 16% of the cells of a glucose-de-repressed culture (containing 3.8×10^7 cells/ml) to pass through the rotor. (*a*) Synchrony index (F_1 for the first division together with cell numbers (●) and glucose concentration (○) in the medium. (*b*) ATPase activity (measured by P_i release) at pH6.8 (●) and at pH8.6 (△) and also assayed at pH8.6 by proton release (○). (*c*) I_{50} values for oligomycin (μg of oligomycin/mg of protein giving 50% inhibition of the oligomycin-sensitive ATPase) at pH8.6 (○). (*d*) Oligomycin-sensitive (■) and oligomycin-insensitive (●) ATPases, both at pH8.6. Vertical lines indicate mid-points in doublings of cell numbers; ATPase activity is expressed as enzyme units/ml of culture.

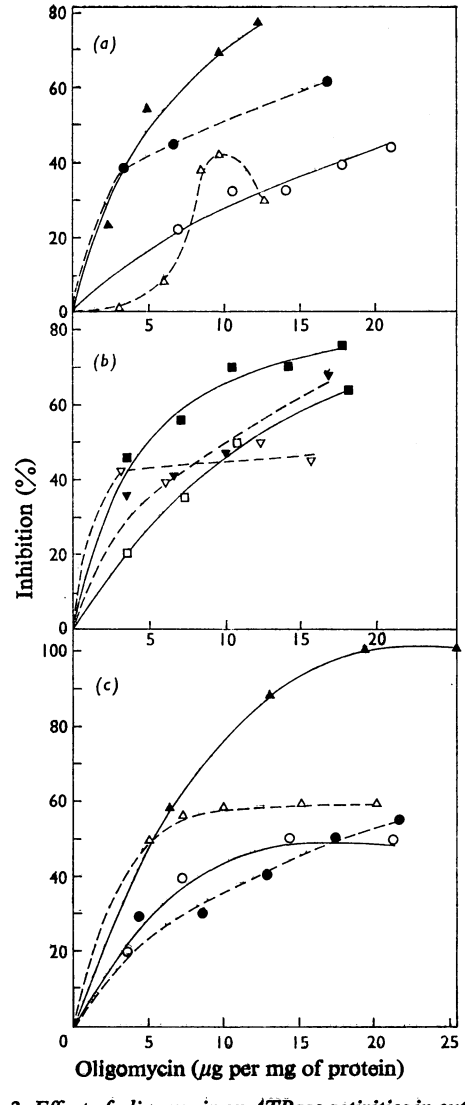


Fig. 3. *Effect of oligomycin on ATPase activities in extracts prepared from S. pombe at different times in the cell cycle*
Sequential additions of small known volumes of oligomycin (2mg/ml in dimethylformamide) were made to ATPase incubation mixtures. After correction for the inhibitory effect of the solvent (10% at 20μl of solvent per 2ml total vol.), percentage inhibition was expressed as a function of inhibitor titres. Titration curves are shown for extracts prepared at times: (*a*) 2.33 (○), 2.67 (●), 3 (△) and 3.33 (▲) h; (*b*) 3.67 (▽), 4 (▼), 4.33 (□) and 4.67 (■) h; (*c*) 5 (○), 5.33 (●), 5.67 (△) and 6 (▲) h; in the experiment shown in Fig. 2.

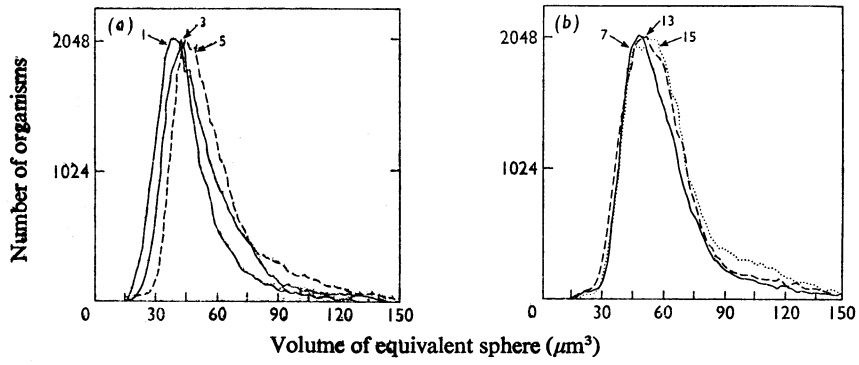


Fig. 4. *Size distributions of cell populations after rate-zonal centrifugation of a glucose-de-repressed exponentially growing culture of S. pombe*

A suspension (50ml) containing 42g wet wt. of cells was prepared after harvesting cells at a population of 4.2×10^7 cells/ml was loaded on a sucrose density gradient in an MSE HSA zonal rotor. Centrifugation was at 1500rev./min for 5min; (0.72×10^4 *g*-min at the sample zone; $\int_0^t \omega^2 \cdot dt = 1.1\times10^4 \text{rad}^2\cdot\text{s}^{-1}$). Size distributions of cells in six fractions (vol. 40ml) were determined in a Coulter Counter model Z_B fitted with a P-64 Channelyzer (64 channels). Settings were as follows: I×A = 1; lower threshold = 10, upper threshold off; matching resistance = 20kohm; edit switch on. The orifice diam. was 70μm. Channels with most organisms contained 2048 counts. Numbers refer to fraction numbers in experiment of Fig. 5. (*a*) Fractions 1, 3 and 5; (*b*) fractions 7, 13 and 15.

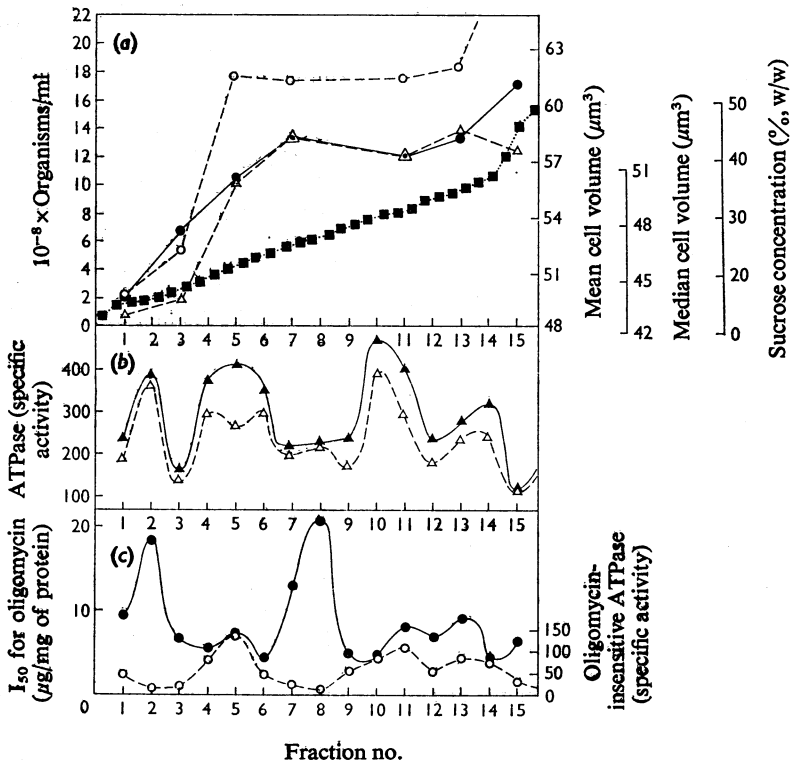


Fig. 5. *Analysis of the cell cycle by rate-zonal centrifugation of cells from a glucose-de-repressed exponentially growing culture of S. pombe*

Culture and centrifugation conditions are described in the caption to Fig. 4. (*a*) Mean cell volumes (●), median cell volumes (△), sucrose density gradient (■) and distribution of cell numbers (○). (*b*) total ATPase (▲) and oligomycin-sensitive ATPase activities (△). (*c*) Oligomycin-sensitivities of ATPase (●) as measured from titration curves (I_{50} values, μg of oligomycin/mg of protein giving 50% inhibition of the oligomycin-sensitive enzyme) and oligomycin-insensitive ATPase (○). ATPase activities are expressed as specific activities (enzyme units per mg of protein).

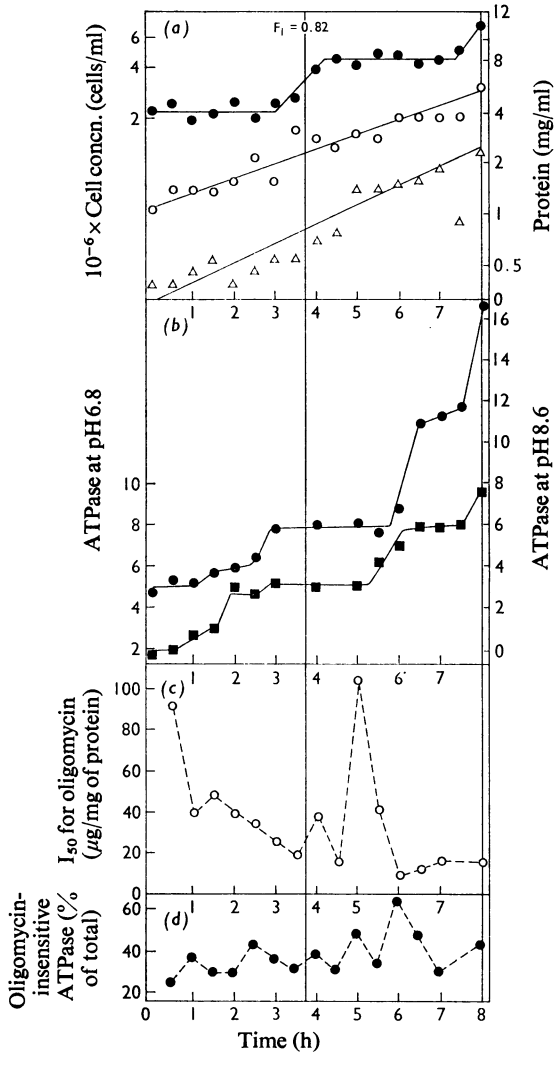


Fig. 6. *Changes in cell population, total cell protein, protein in cell-free extracts, activities of ATPase and sensitivity of ATPase to oligomycin during synchronous growth of S. pombe in the presence of glycerol*

An exponentially growing culture containing 4.9×10^7 cells/ml was subjected to continuous-flow size selection so that 5% of the cells escaped harvesting in the rotor. (*a*) Synchrony index (F_I) for the first doubling in cell numbers (●), protein concentration in 100-fold concentrate of culture (○) and in the cell-free extracts (△). (*b*) ATPase activities at pH8.6 (●) and at pH6.8 (■). (*c*) Oligomycin-sensitivities of ATPase as I_{50} values. (*d*) Oligomycin-insensitive ATPase. Vertical lines indicate mid-points in doublings in cell numbers. ATPase activities are expressed as enzyme units/ml of culture.

The size distributions of cell populations in the fractions obtained after rate-zonal centrifugation of a catabolite-de-repressed exponentially growing cell suspension are shown in Fig. 4. Mean cell volumes and cell numbers in these fractions are shown in Fig. 5(*a*). Total ATPase of cell-free extracts shows a complex pattern of expression; at least three maxima of enzyme activity per cell occurred during the first 0.75 of a cell cycle (Fig. 5*b*). Oligomycin-sensitivity (as measured by values for I_{50}) varied over a fourfold range and showed three maxima over that portion of the cell cycle amenable to study by this method (Fig. 5*c*).

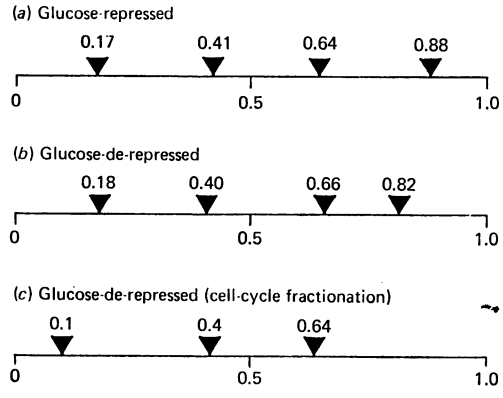
Changes in ATPase activity and oligomycin-sensitivity during synchronous growth in the presence of glycerol

Flocculation of organisms during the late exponential phase of growth of *S. pombe* in the presence of glycerol decreases the yield of organisms available for continuous-flow size selection. In the experiment shown in Fig. 6 only 5% of the original population remained in the rotor effluent; this suspension, however, gave excellent synchrony ($F = 0.82$), with a cell-cycle time (4.2 h) similar to the mean generation time of the exponential culture. Total protein in the culture increased exponentially, and the efficiency of cell disruption remained fairly uniform throughout the experiment (Fig. 6*a*).

ATPase activity increased in a stepwise manner (Fig. 6*b*) and doubled during each cell cycle. At both pH values two steps are clearly evident in the second cycle, and at pH8.6 corresponding steps in the first cell cycle are also seen (mid-points at 0.54 and 0.95 of the cell cycle). A double step is less evident at pH6.8 in the first cycle. Sensitivity to oligomycin (values for I_{50}) measured from titration curves showed a fivefold variation (Fig. 6*c*). Oligomycin-insensitive ATPase accounted for between 25 and 60% of the total ATPase of the extracts and showed regular fluctuations (Fig. 6*d*).

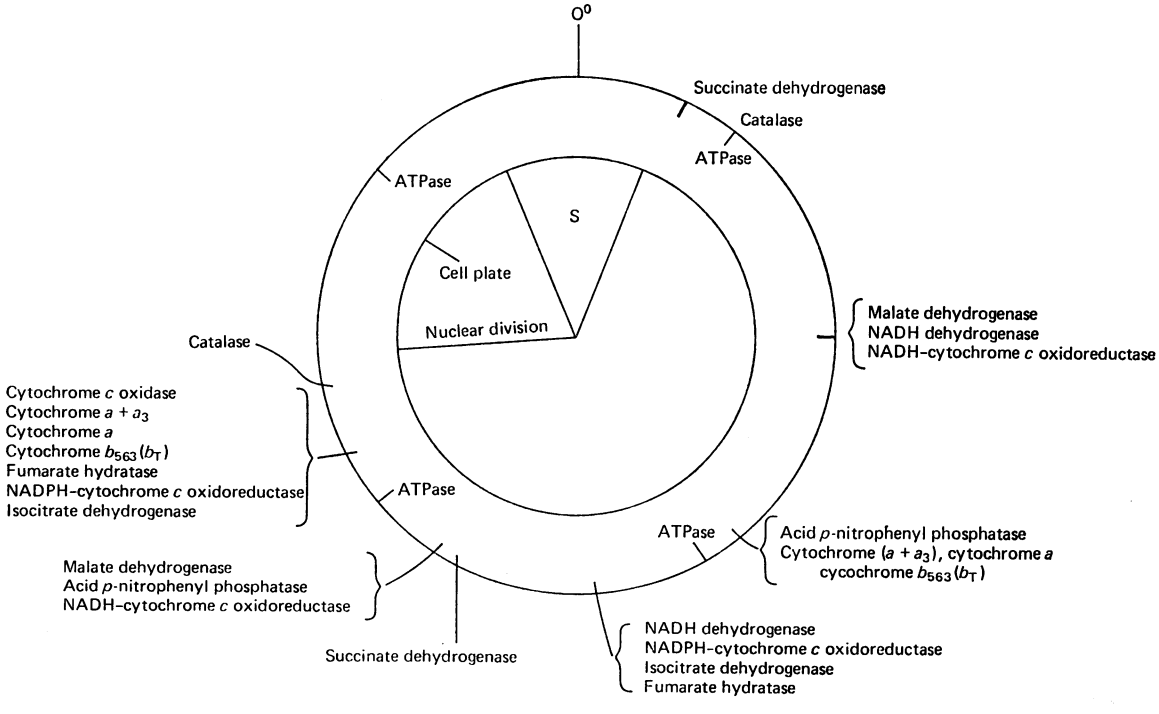
Discussion

Previous results have indicated that many mitochondrial enzymes in the fission yeast *S. pombe* are catabolite-repressible (Poole & Lloyd, 1973; Lloyd & Edwards, 1976). The progress of biogenesis of mitochondrial components in synchronous cultures may be more easily appreciated in a physiological situation where the complex modulation of respiratory activity resulting from glucose repression is completely eliminated, i.e. in a culture growing in the presence of glycerol (Poole & Lloyd, 1974). In both catabolite-repressed and -de-repressed conditions, the activity of ATPase is expressed discontinuously as the organisms of the synchronous culture progress through the cell cycle. Cells growing with glycerol show a double step in the appearance of



Scheme 1. *Cell-cycle maps of timings of maxima of ATPase activities during the cell cycle of glucose-grown S. pombe*
In (*a*) and (*b*), the abscissa represents the cell cycle; 0 and 1.0 are defined by the mid-point of doubling in cell numbers. In (*c*) the resolved portion of the cell cycle in cell-cycle-fractionation experiments is represented as a linear scale and normalized with respect to the measured mean cell volumes in fractions from the rotor. ▼, Timings of maxima in ATPase activities.

ATPase in each cell cycle, whereas cells growing with glucose (under both glucose-repressed and glucose-de-repressed conditions) show a complex oscillatory mode of expression. In this respect ATPase is similar to several other mitochondrial activities; thus respiratory rates, cytochrome *c* oxidase activities and total cytochrome $a+a_3$ also oscillate in glucose-grown cultures, but increase in double steps in the cell cycle of glycerol-grown cells (Poole *et al.*, 1973, 1974; Poole & Lloyd, 1973, 1974). Other mitochondrial enzymes (e.g. succinate dehydrogenase), however, behave quite differently in that they show two maxima in their activities under both catabolite-repressed and catabolite-de-repressed conditions. We suggest that those enzymes that are synthesized under the dual control of nuclear and mitochondrial DNA [cytochrome $a+a_3$, cytochrome b_{563} (b_T), and the mitochondrial ATP synthetase complex] exhibit similar and characteristic patterns of synthesis in synchronous cultures growing under different environmental conditions. Thus both cytochrome oxidase and mitochondrial ATPase are relatively stable enzymes in cells growing with glycerol; discrete periods of synthesis (and/or



Scheme 2. *Cell-cycle 'map' of timings of enzyme activities during the cell cycle of glucose-grown S. pombe*
Data on enzymes are taken from Scheme 1 (the present paper) and from Poole & Lloyd (1973); those on cytochromes are from Poole *et al.* (1974), and those on cell cycle events are from Mitchison (1971). O^0 indicates the midpoint of doubling in cell numbers; S represents the duration of nuclear DNA synthesis.

assembly) are followed by periods in which no alteration of enzyme activity is observed. However, in cultures growing on glucose, synthesis (and/or assembly) is invariably followed by a rapid decline in activity. Whether, in the case of ATPase, this decrease reflects inactivation by dissociation of subunits, binding of an ATPase inhibitor, a conformational change, or loss of enzyme protein owing to a specific process of proteolysis responsible for turnover of the enzyme, presents an important problem which must be tackled before the levels of control operating in this system can be precisely ascertained. It is evident, with the cytochrome oxidase complex, that a decline in capacity for electron transport is accompanied by loss of haem *a* detectable in difference spectra (Poole *et al.*, 1974).

The relative timings of the maxima of ATPase activity in glucose-grown *S. pombe* are summarized in Scheme 1; close agreement between the occurrence of maxima in enzyme activities is evident when glucose-repressed (1*a*) and de-repressed cells (1*b*) are compared, and confirmation of the timings of the first three of these maxima comes from a completely independent method of investigation, that of cell-cycle fractionation (1*c*), which can only be applied to the first 0.75 of a cell cycle. Scheme 2 summarizes all the information now available on the timing of enzyme accumulation during the cell cycle of glucose-repressed cells. The second and third maxima of ATPase activity coincide with the two maxima previously observed for cytochromes *a*, $a+a_3$ and b_{563} (b_T), and the third maximum of ATPase activity (at 0.65 cycle) is close to the single maximum observed for cytochrome *c* oxidase activity (at 0.67 cycle).

A sixfold increase in sensitivity of mitochondrial ATPase to oligomycin has previously been observed during catabolite de-repression of *S. pombe* (Lloyd & Edwards, 1976), and possible factors involved have been discussed. A major contribution in that system is provided by a naturally occurring ATPase inhibitor; binding of this component to the ATP synthetase complex renders it more susceptible to inhibition by a wide variety of agents (Lloyd & Edwards, 1976). Whether the fourfold fluctuation in oligomycin-sensitivity observed in glucose-grown synchronous cultures also reflects binding of a natural ATPase inhibitor remains to be investigated.

Experiments with synchronous cultures prepared by the continuous-flow size selection method provide a powerful approach to the study of the synthesis and assembly of molecular complexes into membranes. It has great advantages both over previous methods of selection synchrony (e.g. selection by rate-zonal centrifugation on density gradients; Mitchison & Vincent, 1965) and other methods of studying mitochondrial development which rely on the use of protein-synthesis inhibitors (Lloyd, 1974). The present method induces no detectable metabolic perturbation, and thus provides a unique opportunity for the study of the temporally organized sequences of events occurring during 'normal' or 'balanced' cell growth.

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