Development of Respiratory Activity during the Cell Cycle of *Schizosaccharomyces pombe* 972 h⁻: Respiratory Oscillations and Heat Dissipation in Cultures Synchronized with 2'-Deoxyadenosine

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Rates of oxygen uptake and heat dissipation were measured in cultures of *Schizosaccharomyces pombe* that had been induced to divide synchronously by adding 2 mM-2'-deoxyadenosine and then removing the inhibitor after 4 h. Respiratory oscillations occurred during the last 1.5 h of treatment with deoxyadenosine and throughout the subsequent period of synchronous growth. Before completion of the first synchronous division three peaks of oxygen uptake occurred, the third peak being coincident with cell division. These peaks were less sensitive to the rate-stimulating effect of the uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone, than were the troughs, so that in the presence of the uncoupler the oscillations were attenuated. In the absence of uncoupler, heat dissipation of the culture increased linearly during and after deoxyadenosine treatment, with sharp increases (approximate doublings) in the rate of dissipation occurring at intervals similar to the mean generation time of an exponential culture. Heat dissipation also increased continuously in samples removed from such a culture and incubated with the uncoupler. The possible modulation of oxygen uptake rates by respiratory control, and the implications of linear increases in heat dissipation are discussed.

INTRODUCTION

Many methods have been developed for the preparation of synchronous cultures of the fission yeast *Schizosaccharomyces pombe* (for review, see Mitchison, 1971). The most extensively used has been the selection of a homogeneous age class of cells from an asynchronous culture by density gradient centrifugation. Such cultures are relatively unperturbed and may be used to investigate the events constituting the 'normal' cell cycle (Kubitschek & Claymen, 1976). However, much may be learned about the temporal control of cell cycle events by studying synchronized cultures in which the normal cycle is distorted. Inhibition of DNA synthesis in *S. pombe* by 2'-deoxyadenosine (dAdo), and its subsequent reversal by removal of the inhibitor, synchronize DNA synthesis and cell division (Mitchison & Creanor, 1971). Sissons, Mitchison & Creanor (1973) have shown that three enzymes that exhibit step patterns of periodic activity in selection-synchronized cultures exhibit continuous increases in activity in dAdo-synchronized cultures. It has been suggested that the synthesis of these enzymes constitutes part of a 'growth cycle' that is not necessarily synchronized with the 'DNA-division cycle', and further, that measurements of growth variables in both selection- and induction-synchronized cultures may permit their assignment to the appropriate cycle (Mitchison, 1973). In contrast, Kramhøft, Nissen & Zeuthen (1976) have reported that two of these enzymes do not exhibit step patterns of activity in either selection-synchronized cultures or in cultures previously synchronized by heat shocks. Only between successive heat shocks were stepwise increases in activity observed.

The development of respiratory activities in selection-synchronized cultures has been investigated (Poole, Lloyd & Kemp, 1973), and these studies are now extended to dAdo-synchronized cultures. The results described in this paper show that oscillations in respiration rate persist in induction-synchronized cultures but the mechanisms involved in their generation are fundamentally different from those proposed previously. Similar experiments with cells growing in complex, rather than defined, growth medium have been reported previously (Poole & Pickett, 1976).

METHODS

Organisms and growth conditions. *Schizosaccharomyces pombe* strain 972h⁻ was maintained and grown on the defined medium that contained 1 % (w/v) glucose as carbon source (Poole *et al.*, 1973) except that biotin in the stock vitamin solution was first dissolved in 50 % (v/v) ethanol, giving a final ethanol concentration of 0.002 % (v/v) in the complete medium (Gutz *et al.*, 1974). Cultures were inoculated to give an initial population of 1×10^5 to 3×10^6 cells ml⁻¹ and were grown in conical flasks that contained one-fifth their volume of medium, shaken at 30 °C and 200 rev. min⁻¹ on a rotary incubator. Where frequent sampling was required, cultures were grown in a Quickfit 2 l fermenter vessel that was agitated magnetically using a Teflon-coated stirrer bar and fitted with two spargers for aeration. As described below, small cultures were sometimes grown in the open reaction vessel of an oxygen electrode apparatus.

Induction of synchrony. 2'-Deoxyadenosine was added as a powder to an exponentially growing culture to give a final concentration of 2 mM. After 4 h (Mitchison & Creanor, 1971) the inhibitor was removed by accelerating the culture in an MSE 18 centrifuge to 15000 g (at $r_{av.}$) at ambient temperature, followed by immediate deceleration. Sedimented cells were resuspended in the same volume of prewarmed fresh medium (lacking dAdo) and replaced in the culture vessel.

Assessment of synchrony. The degree of synchrony of the first division after removing dAdo was compared in separate experiments using the index of Blumenthal & Zahler (1962), and calculated from the equation:

$$F_1 = (N/N_0) - 2^{1/g}$$

N_0 and N are, respectively, the numbers of cells immediately prior to and after the period of division (of duration t) and, in these experiments, g is the period between removal of the inhibitor and the mid-point of the first division. It may be inappropriate to use the term 'cell cycle' to describe either this period or the period between the two synchronous divisions, since the events constituting them do not form a repetitive sequence. Also, the beginning of g is not marked by a synchronous cell division, unlike the conventions of cell cycle analysis.

Cell numbers. These were determined in samples fixed with formalin (final concentration 1 %, v/v) using a counting chamber with improved Neubauer rulings (Hawksley, Lancing, Sussex) applying the criteria of Mitchison (1970). Alternatively, a Coulter counter model Z_{BT} fitted with a probe having an aperture diameter of 50 µm was used. These methods gave comparable results.

Measurements of oxygen uptake. Oxyten uptake rates in 2 ml samples of culture were measured with an O₂ electrode (Rank Bros, Bottisham, Cambridge) using the calibration of Chappell (1964). Respiration rates were also measured on 2 ml samples, which had been concentrated by filtration through Oxoid Nuflow membranes (0.45 µm pore size), in an O₂ electrode system open to the atmosphere, as described by Poole (1977). In a control experiment, air-saturated water (2 ml) instead of culture was incubated for 6 h in the open electrode vessel. Fluctuations in the recorded O₂ tension were less than 4 % of the mean over this period; drift was less than 1 % per h. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was used as a methanolic solution (1 mg ml⁻¹). Volumes of addition were such that the final methanol concentration never exceeded 0.7 % (v/v), and appropriate controls were performed to allow for the effects of methanol alone on respiration rates.

Measurements of heat dissipation. An LKB 2107 microcalorimetry system, fitted with a stainless steel flow cell (vol. 1.2 ml), was used. For continuous monitoring, a growing culture was pumped at 53 ml h⁻¹ to a T-piece using an LKB 10200 Perplex peristaltic pump. An LKB 12000 Varioperpex pump was used at the effluent line from the calorimeter to draw culture and air (from the T-piece) through the flow cell at a total flow rate of 77 ml h⁻¹. This produced a regular, segmented flow of culture and air through the apparatus. To investigate heat dissipation in the presence of CCCP, samples (10 to 40 ml) removed from the culture were mixed with the uncoupler at 30 °C and immediately pumped into the calorimeter as described, until a steady increase in heat dissipation was recorded. Between samples, the flow cell was flushed through with medium. The time for the passage of culture into the flow cell and the beginning of the calorimetric response was about 3 min, during which the culture did not become anoxic. The temperature of the thermostatic air bath was 30 °C; a slow flow of water at 20 °C was used to assist dissipation of heat from the instrument. The calorimetric response was amplified with a microvolt ammeter (30 or 100 µV range) and was recorded

Fig. 1

Fig. 2

Fig. 1. Effect of CCCP on oxygen uptake in cell suspensions from a dAdo-synchronized culture of *S. pombe*. The inhibitor was added to an exponential culture at zero time and, after 4 h incubation, removed by centrifugation; cells were resuspended in fresh medium lacking dAdo. F_1 is the synchrony index of the first synchronous doubling (mid-point indicated by vertical line) in cell numbers (●). Oxygen uptake measurements on culture samples removed at approx. 10 min intervals were made in the absence (○) or in the presence of $9.8 \mu\text{M}$ -CCCP (□).

Fig. 2. Oxygen uptake in a dAdo-synchronized culture of *S. pombe* using the open oxygen electrode. To an exponentially growing culture, dAdo (2 mM) was added at the first arrow (zero time) and removed by centrifugation at the second arrow. F_1 denotes the synchrony index of the first synchronous doubling (mid-point indicated by vertical line) in cell numbers (●). Three samples of culture were removed, concentrated by rapid filtration and then cultured in an open O_2 electrode vessel. The degrees of concentration were 20-fold (0.35 to 2.25 h), 10-fold (2.4 to 4.0 h) or 3.3-fold (4.4 to 8.0 h); dashed lines indicate the change to a less concentrated cell suspension. The respiration rates (○) have been corrected for the varying degrees of concentration.

on a potentiometric recorder, with a full-scale deflexion of 100 mV. The calorimeter was calibrated at the end of each experiment, with aerated medium flowing through the cell, by applying an internal current of 1 to 4 mA for 1000 s from an LKB 10700 microcalorimetry control unit, to achieve a steady state.

Extinction of cultures. This was measured at 600 nm in a specially constructed flow-through cell of 1 cm light path in a recording spectrophotometer. The culture was pumped at 160 ml h^{-1} through the cell by an LKB 10200 peristaltic pump and then returned to the culture vessel.

Chemicals. 2'-Deoxyadenosine and CCCP were from Sigma.

RESULTS

Oxygen uptake in dAdo-synchronized cultures

After adding dAdo to an exponentially growing culture, cell numbers continued to increase for 2 to 3 h; in eight experiments the mean increase in numbers was twofold (S.D. ± 0.3 -fold). After removal of the inhibitor at 4 h, cell numbers remained constant for a further 1.5 h prior to the first synchronous division (Figs 1 and 2). The interval between removal of dAdo and the mid-point of the first synchronous division was 2.8 h (S.D. ± 0.2 ; 8 experiments). A second synchronous division followed, its mid-point occurring 1.4 h (S.D. ± 0.3 ; 4 experiments) after the mid-point of the first division. This period is therefore substantially shorter than the length of the normal cell cycle in selection-synchronized cultures (about 2.7 h). These results with strain 972 h⁻ and a 4 h pulse of dAdo are essentially the same as those described by Mitchison & Creanor (1971) for strain 132 and a 3 h pulse of dAdo.

During the pulse with dAdo, respiration rates approximately doubled and exhibited oscillations of small amplitude during the last 1.5 h of the pulse (not shown in Fig. 1, but see Fig. 2). After removal of the inhibitor, respiration rates measured in the absence of CCCP oscillated markedly, rising to three peaks in the period before completion of the first synchronous division (Fig. 1). The amplitude of the oscillations (i.e. peak to trough) varied from 9 to 45 % of the total respiration measured at the peaks. Oscillations persisted at least 4 h after removal of the inhibitor but those occurring after the first synchronous division were not studied further.

As the observed fluctuations could reflect inadequacies of the experimental technique, particularly with regard to infrequency and errors in sampling, respiration rates were then studied in the open O_2 electrode system, thus allowing a continuous recording of O_2 tension and more frequent calculation of O_2 uptake rates. Such an experiment (Fig. 2) confirmed the pattern of oscillations described; oscillations started 90 to 100 min before the end of the dAdo pulse and then exhibited three maxima before completion of the first synchronous division. The mean timings of the three peaks in each of four experiments (including one using the open electrode system) are shown in Fig. 3. When the time of removal of dAdo is designated 0, and the mid-point of the first synchronous division is designated 1.0, the first two peaks occur at 0.4 and 0.75; the third peak is almost coincident with the mid-point of the first doubling in cell numbers.

Respiration rates (per cell) appeared consistently higher when measured using the open O_2 electrode than when the conventional, closed system was used (see Figs 1 and 2). This may be due to the failure of cells, which were removed from a growing culture and transferred to a vessel where the sample was not in contact with O_2 , to attain their potential steady-state rate of respiration within the time in which measurements were made (about 3 to 5 min). This problem has been discussed in greater detail by Degn, Lilleør & Iversen (1973).

Effects of CCCP on respiration rates during the dAdo pulse and subsequent synchronous growth

Preliminary experiments with cells from exponentially growing cultures confirmed that maximum stimulation of respiration rates (70 to 100 %) was obtained with 10 to 20 μ M-CCCP (Poole *et al.*, 1973). During synchronous growth, minimum rates of respiration were preferentially sensitive to the rate-stimulating effect of 9.8 μ M-CCCP (Fig. 1), so that in its presence the oscillations were attenuated. The mean stimulations obtained in the experiment shown in Fig. 1 were 59 % (S.D. $\pm$ 9) at the peaks and 91 % (S.D. $\pm$ 15) at the troughs. Lowering the concentration of CCCP to 4.9 μ M gave 28.4 % (S.D. $\pm$ 3) and 45.2 % (S.D. $\pm$ 12) at peaks and troughs respectively (not shown). These results contrast with the effects of CCCP on culture samples withdrawn from synchronous cultures prepared by selection, in which the respiratory maxima, rather than minima, were preferentially sensitive to stimulation by CCCP (Poole *et al.*, 1973).

Since significant stimulation of respiration rates of asynchronous cultures by CCCP occurs over a narrow range of concentrations, and concentrations greater than about 40 μ M inhibit respiration, it was important to establish that optimum concentrations of uncoupler were used at peaks and troughs. Culture samples removed both during the dAdo pulse (Fig. 4a) and during the subsequent synchronous growth (Fig. 4b) were titrated with CCCP. Before addition of dAdo and up to 20 min after addition, the maximum stimulation of respiration elicited by 10 to 20 μ M-CCCP was 100 to 110 %. After 1 h incubation with dAdo, respiration was stimulated only 20 % by 40 μ M-CCCP. Subsequently, sensitivity to CCCP increased during the pulse. The degree of stimulation by CCCP of the respiration rates at the first two minima after removing the inhibitor exceeded by 2.5 to 3.0-fold the stimulation at the first two maxima (Fig. 4b). Maximum stimulation was elicited by similar concentrations of CCCP in both cases. These results are consistent with the

Fig. 3. Maps of timings of peaks in O_2 uptake during the period between the end of a 4 h pulse with dAdo (o) and the mid-point of the first synchronous doubling in yeast numbers (1.0). Timings of the peaks of O_2 uptake were measured with the open O_2 electrode system (▽) or by removing samples from a larger culture at approx. 10 min intervals and analysing with a conventional closed O_2 electrode apparatus (▼). Arrows with crossbars indicate the mean values with standard deviations in four separate experiments. The three points shown in each horizontal row were determined in one experiment.

Fig. 4. Effect of CCCP on respiration rates of cells in samples removed from cultures during a pulse with dAdo (a) and the subsequent synchronous growth (b). The effects of CCCP are shown for samples withdrawn 0.3 h (●), 1.1 h (○) and 3.75 h (□) after adding 2 mM-dAdo to an exponentially growing culture, and for the first (▲) and second (△) troughs and the first (▼) and second (▽) peaks in the period following removal of dAdo.

modulation of respiration rates during synchronous growth by respiratory (acceptor) control, i.e. the rate limitation of O_2 uptake by the availability of ADP in phosphorylating respiratory chains (Chance & Williams, 1956).

Stimulation of heat dissipation by CCCP in exponential cultures

The addition of CCCP to an exponentially growing culture substantially stimulated heat dissipation. The recorder traces obtained were similar to those published previously (Poole & Haddock, 1975) but a steady-state level of heat dissipation was not attained because of continued growth of the organisms. The degree of stimulation by CCCP was calculated after extrapolation of both stimulated and unstimulated rates of heat dissipation to the time at which CCCP was added. Maximum stimulations of both respiration rate and of heat dissipation (Fig. 5) were elicited by similar concentrations of CCCP, although optimum stimulation of respiration rate was approximately five-fold greater than the stimulation of heat dissipation. This presumably reflects the relatively small contribution of heat dissipation from coupled electron transport to the total in a growing culture of a highly fermentative organism. Nevertheless, fluctuations in heat dissipation that may accompany oscillations in oxygen uptake should be of sufficient magnitude (up to 10% of the total) and of low frequency (about 1 h) to be detectable by continuous-flow microcalorimetry.

Fig. 5

Fig. 5. Stimulation by CCCP of respiration rates and dissipation of heat of cells from an exponentially growing culture. Results for respiration rates (●) and heat dissipation (○) are pooled from four and five separate experiments respectively, in which the cell population varied from 0.7×10^7 to 3.1×10^7 organisms ml^{-1} .

Fig. 6

Fig. 6. Heat dissipation of a dAdo-synchronized culture of *S. pombe*. Heat dissipation was measured continuously (—) using a flow microcalorimeter. The vertical line indicates the mid-point of the first synchronous doubling in cell numbers (●). F_1 denotes the synchrony index of this division. dAdo (2 mM) was added at zero time (indicated by the first arrow) and removed by centrifugation at the second arrow.

Heat dissipation during the dAdo pulse and after removal of the inhibitor

When an exponentially growing culture was pumped to the calorimeter, the calorimetric response rapidly increased above the level produced by uninoculated medium alone (Fig. 6). After equilibration of the culture within the flow cell (approx. 15 min), the rate of heat dissipation gradually increased until the addition of dAdo. It then increased approximately linearly during the first 2.8 h of the pulse at a rate of about $0.18 \text{ J h}^{-1} \text{ h}^{-1}$; the rate of increase then changed to about $0.31 \text{ J h}^{-1} \text{ h}^{-1}$. After resuspension of cells in fresh medium, heat dissipation again increased linearly. At 1.5 h after resuspension (2.7 h after the previous sharp increase), the rate suddenly increased 1.8-fold. There was no evidence for oscillations in heat dissipation, three experiments giving similar results. In contrast, heat dissipation increased exponentially in an asynchronous culture (results not shown), doubling in the same interval as the mean generation time.

Heat dissipation of samples, incubated with CCCP, from dAdo-treated cultures

In the presence of CCCP, heat dissipation (Fig. 7) increased continuously in samples withdrawn both during the dAdo pulse and during the subsequent synchronous growth. The doubling time for heat dissipation was 2.9 h, identical to the length of the period between removal of dAdo and the mid-point of the first division. The precision of this method, which suffers from relatively infrequent sampling, is probably inadequate to distinguish between an exponential increase and a series of linear increases. Although heat dissipation in the absence of CCCP was not measured using this sampling technique, both methods should give similar results, since O_2 is not depleted during the flow to the calorimetric cell and growth temperature is maintained throughout. The rate of increase in absorbance of the culture (Fig. 7) was clearly greater than linear. In agreement with the results of Mitchison & Creanor (1971), the rate of increase between removal of dAdo and the first synchronous division was less than that during the inhibitor pulse.

Fig. 7. Heat dissipation in the presence of $12.3 \mu\text{M}$ -CCCP of samples removed from a dAdo-synchronized culture of *S. pombe*. Each sample (12 ml) was mixed with CCCP at 30°C and immediately pumped to the flow cell of a microcalorimeter until a continuous trace of heat dissipation was recorded (○). The vertical line indicates the mid-point of the first synchronous doubling in cell numbers (●). F_1 denotes the synchrony index of this division. dAdo (2 mM) was added at zero time (indicated by the first arrow) and removed by centrifugation at the second arrow. Extinction of the culture (----) was measured continuously in a flow-through cell.

DISCUSSION

This study shows that oscillations in the rates of oxygen uptake occur in cultures synchronized by pulsing with dAdo. These oscillations differ, however, in several respects from those previously described in selection-synchronized cultures (Poole *et al.*, 1973). First, the timing and periodicity of the oscillations differ. In selection-synchronized cultures two peaks occur in each cell cycle and their periodicity is 0.5 of a cycle (about 1.35 h), whilst in the present study three peaks, separated by 0.8 to 1.0 h, occur between removal of the dAdo and completion of the first division. Oscillations are first observed prior to this, at about the same time as spontaneous recovery of DNA synthesis begins (Sissons *et al.*, 1973). Secondly, the control mechanisms responsible for their generation appear to be quite different. The response of the oscillations to CCCP in the present study are consistent with the modulation of respiration rates by a respiratory (acceptor) control mechanism. That is, the oxygen uptake minima reflect the rate limitation of respiration by availability of ADP (state 4), which is relieved by an uncoupler of oxidative phosphorylation. The maximum rates of respiration approach the uncoupled rates, being limited by the capacity of the respiratory chain for electron transport (state 3; Chance & Williams, 1956). Confirmation of this hypothesis would involve measurements on mitochondria isolated from cells at stages of the cycle at which respiration was at a maximum or minimum. The time required for the preparation of mitochondria from this organism (Poole & Lloyd, 1976) and the poor respiratory control that they exhibit (R. K. Poole and D. Lloyd, unpublished results) make the success of this approach doubtful.

Synchronous cultures of *Candida utilis*, growing with either glucose or acetate as carbon source (D. Lloyd and R. K. Poole, unpublished results; Lloyd, 1974) and of *Alcaligenes eutrophus* (Edwards & Jones, 1977) also exhibit respiratory oscillations in which the minima are preferentially stimulated by CCCP. Variations in the ability of CCCP to relieve respiratory control may reflect the changing energy requirements of the organism during its cell cycle.

The linear increase in heat dissipation and the oscillating rates of oxygen uptake result

in a fluctuation in the ratio of heat dissipated per unit of oxygen consumed. Possible explanations of this are that it reflects the changing efficiency of oxidative phosphorylation or the varying contribution of glycolysis to energy metabolism (Poole & Haddock, 1975) during synchronous growth.

Unlike total RNA synthesis and increases in enzyme activities (Sissons *et al.*, 1973), heat dissipation appears to be synchronized by dAdo, exhibiting a series of linear increases rather than an exponential increase. In previous experiments with selection-synchronized cultures (Poole *et al.*, 1973), a near-linear increase in heat dissipation was also observed in one complete cell cycle, but the points at which the expected doubling in rate occurred were not clearly defined. The finding that heat dissipation increases linearly rather than exponentially in synchronized cultures of *S. pombe* is of interest in view of the recent observation by Kubitschek & Claymen (1976) that the uptake of glucose and glycine increases linearly. Both results support the hypothesis of the generality of linear, rather than exponential, modes of growth during the cell cycle (Kubitschek, 1970). The accuracy afforded by continuous-flow microcalorimetric measurements of overall metabolic activity may be useful in evaluating this hypothesis, since the maximum deviation between variables that double with linear or exponential kinetics is less than 6%.

The argument developed by Mitchison (1973) and his collaborators would lead to the conclusion that both respiratory oscillations and heat dissipation are associated with the 'DNA-division' cycle, since increases in these variables are synchronized in cultures prepared either by size selection or dAdo-induction. However, since the physiological bases for respiratory oscillations in these two kinds of culture appear to be fundamentally different, this interpretation is inappropriate. Finally the possibility cannot be excluded that the synchronization method used here (including centrifugation of cells and resuspension in fresh medium) may itself generate the respiratory oscillations. This seems unlikely because their expression is entrained to specific points in the cycle; decreasing the length of the cycle by growth in complex medium has no effect on the relative timing of the peaks (Poole & Pickett, 1976).

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