

## CHANGE IN THE RATE OF CO<sub>2</sub> PRODUCTION IN SYNCHRONOUS CULTURES OF THE FISSION YEAST *SCHIZOSACCHAROMYCES POMBE*: A PERIODIC CELL CYCLE EVENT THAT PERSISTS AFTER THE DNA-DIVISION CYCLE HAS BEEN BLOCKED

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### SUMMARY

CO<sub>2</sub> production has been followed by manometry in synchronous and asynchronous cultures of *Schizosaccharomyces pombe* prepared by elutriation from the same initial culture. The rate of production follows a linear pattern in synchronous cultures with a rate change once per cycle at the time of cell division. This pattern is most clearly shown in oscillations of the difference between values of the second differential (acceleration) for the synchronous and asynchronous cultures. The association between the rate change and the time of division is maintained during growth speeded up in rich medium and slowed down in poor medium and at lower temperature. It is also maintained after a shift-up in temperature. Results with *wee* mutants suggest that the association is with the *S* period rather than division itself. The rate and acceleration of CO<sub>2</sub> production are approximately proportional to cell size (protein content) in asynchronous cultures.

When synchronous cultures of the temperature-sensitive mutants *cdc2.33* and *cdc2.33 wee1.6* are shifted up to the restrictive temperature, the DNA-division cycle is blocked. The oscillatory pattern of CO<sub>2</sub> production, however, continues for one to two cycles until the acceleration reaches a constant value, after which the oscillations are undetectable. This point is reached later in the double mutant and there is a phase difference in the oscillations compared to those in the single mutant. With both blocked mutants the 'free-running' oscillations are about 15% shorter than the normal cycle time. There are well-known examples of such oscillations in eggs but they are rare in growing systems.

### INTRODUCTION

Creanor (1978a) measured CO<sub>2</sub> evolution in synchronous cultures of *Schizosaccharomyces pombe* and found a periodic change in the rate of evolution once per cell cycle. He also blocked the DNA-division cycle with chemical inhibitors (deoxyadenosine and mitomycin C) and found that the periodic changes continued for one

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to two cell cycle times after the blocks. This important finding was one of the earliest unequivocal demonstrations that periodic cell cycle events can continue in growing eukaryotic cells after a block to DNA synthesis and division.

We have extended and refined these measurements using another strain of *S. pombe*, a different medium, a different manometric technique and an improved method of preparing synchronous cultures which also permits an asynchronous control to be measured at the same time. We have also used *cdc* mutants to impose the DNA-division block since they can be more effective and longer lasting than the chemical inhibitors that are available for yeast.

Our data are more extensive and more precise than those of Creanor but they largely confirm his findings except for the exact nature of the periodic change. They also show that the periodic changes continue in blocked mutants for up to two cycles though the timing of these cycles is somewhat shorter than the normal cell cycle. Evidence from size mutants suggests that the position of the periodic change in unblocked cells is associated with the *S* period rather than with mitosis or with the attainment of a critical size.

## MATERIALS AND METHODS

### *Strains*

The wild-type (WT) strain 972h<sup>-</sup> was originally obtained from Professor U. Leupold, Bern. The mutants *cdc2.33* and *cdc10.129* are temperature-sensitive conditionals that form colonies at 25°C but not at 35°C (Nurse *et al.* 1976). The *cdc2*<sup>+</sup> function is required both for mitosis and for the initiation of DNA synthesis (Nurse & Bisset, 1981) but in normal growing cultures only the mitotic block is apparent in *cdc2.33* since nearly all of the cells are in *S* and *G*<sub>2</sub> and they arrest in *G*<sub>2</sub> after a shift-up in temperature. The *cdc10*<sup>+</sup> function is required for the initiation of DNA synthesis. The mutant *wee1.6* divides at about half the size of the wild-type strain (Nurse *et al.* 1976). *wee1.302* is partially defective in the *wee1* gene product and is intermediate in size between *wee1.6* and wild-type (Fantès, 1981). The double mutant *cdc2.33 wee1.6* was constructed by the appropriate crosses and tetrad analysis (Benitez *et al.* 1980).

### *Media*

The standard medium used for most experiments was EMM3, a minimal medium with phthalate buffer (Creanor & Mitchison, 1982). In some experiments a richer medium was used (EMM+YE) in which EMM3 was supplemented with 5 g l<sup>-1</sup> yeast extract. In others, a medium was used (EMM+Asp) in which the NH<sub>4</sub>Cl in EMM3 was replaced by aspartic acid at 5 g l<sup>-1</sup> and the pH adjusted to 5.4.

### *Synchronous cultures*

Cultures were grown up overnight in a shaking water bath at 25°C to 35°C to a cell density of 4 × 10<sup>6</sup> to 6 × 10<sup>6</sup> cells ml<sup>-1</sup>. Synchronous cultures were prepared from these overnight cultures by selecting the smallest cells in an elutriating rotor (Creanor & Mitchison, 1979). These cells were about 1% of the whole population. The remaining 99% of the cells were used to prepare a control asynchronous culture, which was used for manometry at the same time as the synchronous culture.

### *Manometry and other techniques*

CO<sub>2</sub> production was measured in a Warburg manometer using the direct method (Umbreit *et al.* 1964). A 3 ml sample of culture was put into a 15 ml flask and kept there for up to 8 h, and shaken in

a warm water bath. Pressure readings were taken every 5 min and the pressure was relieved every 15 min by opening the manometer valve. Four manometers were normally used, two containing samples from the synchronous culture, one containing a sample from the asynchronous control and one as a thermobarometer. Corrections were made in the usual way for the thermobarometer readings and the flask constants.

This method of measurement ignores the O<sub>2</sub> consumption. Since, however, this yeast has the high respiratory quotient of 10.5 (Hamburger *et al.* 1977) the effect is relatively small: an underestimate of CO<sub>2</sub> production by about 10%. Very large changes in the O<sub>2</sub> consumption during the cycle could distort the apparent CO<sub>2</sub> patterns. This, however, is most unlikely since it is known that O<sub>2</sub> consumption is continuous through the cycle with two small changes in rate (Creanor, 1978*b*).

Cell numbers were measured with a Coulter Counter (Industrial model D) with a 100 µm aperture. Protein was determined by the method of Lowry *et al.* (1951).

### *Analysis and smoothing*

Although the amount of CO<sub>2</sub> produced by a sample was a monotonically increasing function of time, the difference between successive readings was not. It was therefore necessary to smooth the experimental data in order to detect the overall patterns.

In the case of the rate of production curves in Fig. 1, a second-order polynomial was fitted by the method of least squares to 15 experimental readings, seven before and seven after the central point. The rate of production at the central point was taken as the first derivative of the fitted curve at that point. This procedure was repeated for successive central points. As with running means, data points were lost at the beginning and end of experiments.

In the case of the acceleration curves in Figs 2, 3 and 4, the same procedure of curve fitting was carried out except that 21 points were used rather than 15 and the points were not the experimental manometer readings but the differences between successive readings. Only one smoothing process was therefore done to derive the acceleration curves.

## RESULTS

The rates of CO<sub>2</sub> production, plotted on a linear scale, are shown in Fig. 1 for two experiments with WT cells. In each experiment there was a synchronous culture and an asynchronous culture both made from the same starting culture and both having gone through the elutriating rotor (see Materials and Methods). The cell numbers for Fig. 1C,D are shown in Fig. 2, which is from the same experiment. The cell numbers for the other experiment (Fig. 1A,B) are not shown but they were very similar. The lower arrow in the synchronous cultures marks the time of the mid-point of the step in numbers at the end of each cell cycle.

The rate of CO<sub>2</sub> production follows an exponential curve of increase in the asynchronous controls, but there are definite signs of a periodic cell cycle pattern in the synchronous cultures. These signs are consistent with a linear pattern at the single cell level in which there is a constant rate of increase of CO<sub>2</sub> production with a sharp doubling in the rate at the end of the cycle. Lines can be drawn through the points for each cycle and these are partially shown in Fig. 1B. They have been omitted in Fig. 1D so that the reader can judge for himself. The timings of the rate change points are shown by the upper arrows. They occur very near the end of the cycle.

Although there are indications of a linear pattern, it is clear that the curves do not in general show a sharp increase in rate between one linear segment and the next. Instead, the increase is spread over an appreciable period, as shown at the end of the

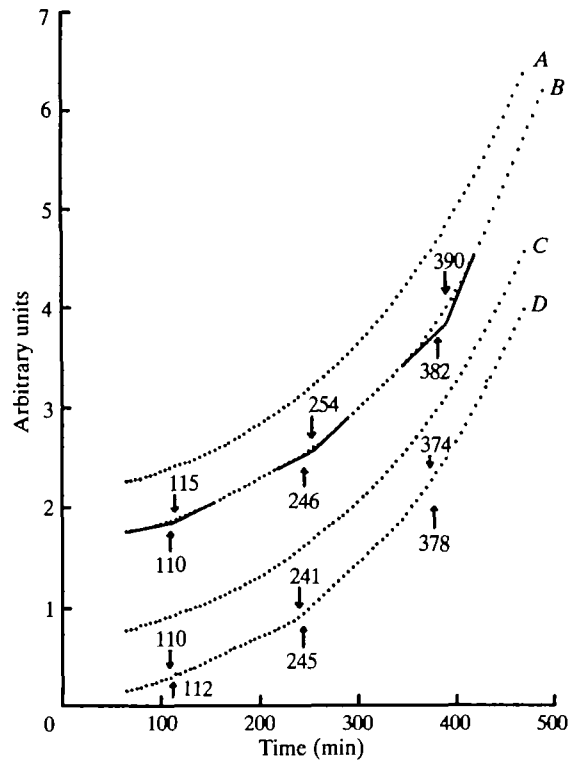


Fig. 1. Linear plot of the rate of  $\text{CO}_2$  production in synchronous and asynchronous cultures of WT cells in EMM3 at  $35^\circ\text{C}$ . Curve A, asynchronous; curve B, synchronous (same experiment as A). See text (Results) for explanation of continuous lines. Curve C, asynchronous; curve D, synchronous (same experiment as C). Upper arrows show the rate change points timing in min. Lower arrows show the mid-points of the cell number steps. Rate of  $\text{CO}_2$  production ( $\mu\text{l CO}_2 \text{ min}^{-1}$ ) in each sample is value in arbitrary units  $\pm$  the following correction factor (in  $\mu\text{l CO}_2 \text{ min}^{-1}$ ): curve A,  $-1.8$ ; B,  $-1.2$ ; C,  $-0.2$ ; D,  $+0.4$ . Each sample was 3 ml and started with  $8 \times 10^5$  cells  $\text{ml}^{-1}$ .

third cycle in Fig. 1B. Even if the rate increase is sharp in individual cells, this spread is to be expected both because of the imperfect synchrony and because of the smoothing process that has been used in generating these curves. We have therefore used a more sensitive measure of curve shape. Instead of calculating the first differential of  $\text{CO}_2$  production (the rate shown in Fig. 1) we have calculated the second differential (the rate of the rate), which we call the 'CO<sub>2</sub> acceleration'. A linear pattern in rate, as in Fig. 1, should show a much more conspicuous stepwise pattern in acceleration even though the steps will still be somewhat smeared by the imperfections of synchrony and by smoothing.

Fig. 2 shows acceleration and number curves for the same experiment as in Fig. 1C,D. The steps in the acceleration curve for the synchronous culture (Fig. 2D) are much more clearly defined than the linear pattern in the rate curve (Fig. 1D) and they support the conclusion that there are doublings in rate at the end of the cycle. In contrast, there are no such steps in the asynchronous control (Fig. 2B). Fig. 2A is a plot of the difference between the acceleration curves for the

synchronous and the asynchronous cultures. It shows marked oscillations with the mid-point of each rise being coincident with the mid-point of the acceleration steps in the synchronous culture.

Fig. 3 is a similar presentation of an experiment with the mutant *cdc2.33*. It was grown up overnight at the permissive temperature of 30°C and then shifted up to the restrictive temperature of 36.5°C immediately after selection on the elutriator. After the shift-up, there was no further cell division in the 'synchronous' culture and no increase in cell numbers (Fig. 3E). The cells, however, continued to grow and elongate for at least 7 h. In the asynchronous control, some of the cells were past the transition point at 0.69 of the cycle (near the time of mitosis) and they went through division before they were blocked in the next cycle. This accounts for the rising cell

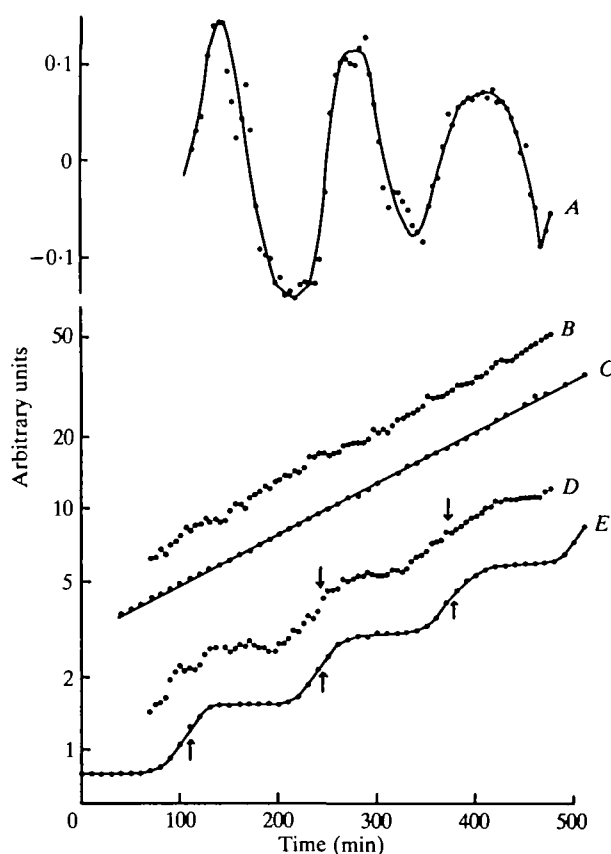


Fig. 2. Acceleration of CO<sub>2</sub> production in a synchronous and an asynchronous culture of WT cells in EMM3 at 35°C (same experiment as in Fig. 1 curves C and D). Curve A, difference between acceleration curves D and B. Ordinate,  $\ln D - \ln B$ . Curve B, acceleration in asynchronous culture; 1 a.u. (arbitrary unit) =  $6 \times 10^{-4} \mu\text{l CO}_2 \text{ min}^{-2}$ . Curve C, cell number in asynchronous culture; 1 a.u. =  $2.1 \times 10^5 \text{ cells ml}^{-1}$ . Curve D, acceleration in synchronous culture; 1 a.u. =  $2 \times 10^{-3} \mu\text{l CO}_2 \text{ min}^{-2}$ . Curve E, cell number in synchronous culture; 1 a.u. =  $10^6 \text{ cells ml}^{-1}$ . Upper arrows show the mid-point of the acceleration steps and the lower arrows show the mid-point of the cell number steps.

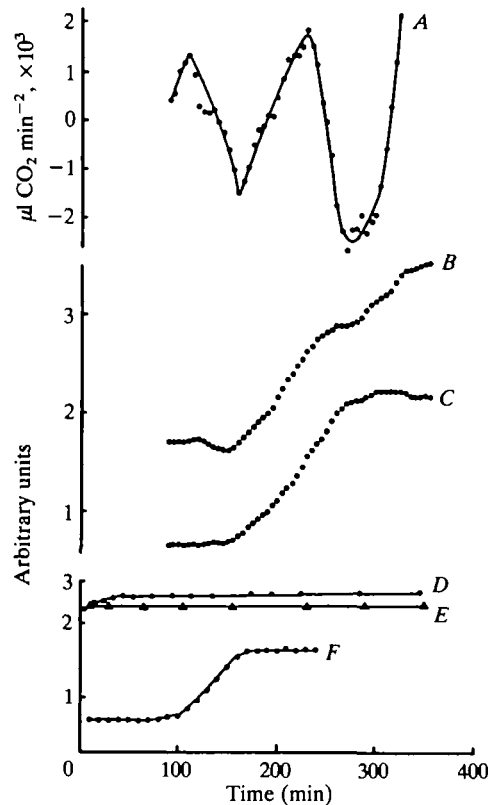


Fig. 3. Acceleration of  $\text{CO}_2$  production in a synchronous and an asynchronous culture of the mutant *cdc2.33* in EMM3 shifted at time zero from  $30^\circ\text{C}$  to  $36.5^\circ\text{C}$ . Curve A, difference between acceleration curves (B and C). Curve B, acceleration in synchronous culture; value in  $\mu\text{l CO}_2 \text{ min}^{-2} = (\text{a.u. value} - 1.0) \times 10^{-2}$ . Curve C, acceleration in asynchronous culture; value in  $\mu\text{l CO}_2 \text{ min}^{-2} = \text{a.u.} \times 10^{-2}$ . Curve D, cell number in asynchronous culture; 1 a.u. =  $3.3 \times 10^5 \text{ cells ml}^{-1}$ . Curve E, cell number in synchronous culture; 1 a.u. =  $3.3 \times 10^5 \text{ cells ml}^{-1}$ . Curve F, cell number in synchronous culture kept at  $30^\circ\text{C}$ , 1 a.u. =  $10^6 \text{ cells ml}^{-1}$ .

numbers during the first hour (Fig. 3D). A portion of the synchronous culture was kept at  $30^\circ\text{C}$ . This is shown in Fig. 3F and gives a measure of the degree of synchrony after selection in the elutriator. The synchronous culture was referred to in inverted commas above because it was not a normal synchronous culture with periodic bursts of division. It was, however, synchronous in cell size and with respect to  $\text{CO}_2$  production, as will be shown, so the inverted commas will be omitted in future.

The acceleration curves in Fig. 3B,C fall into three parts. There is an initial lag phase lasting until 160 min after shift-up in which there is little change in acceleration. This is followed by a middle period in which there are fluctuations in the acceleration, which are more marked in the synchronous culture than in the asynchronous control. Finally, there is a plateau period at the end, which starts in the control at about 270 min and later, if at all, in the synchronous culture. Similar

plateaux have been found in other cell properties in blocked mutants and are discussed later. In general, small cells show a plateau later than large cells. This could account for the difference between the time of appearance of the plateaux here, since the cells in the synchronous culture were smaller on average than those in the control.

It is not easy to detect the patterns in the acceleration curve of the synchronous culture but they become apparent in the difference curve in Fig. 3A. There is a peak and two troughs, which fall within the middle period and are about the same in size and timing as those in the WT synchronous culture (Fig. 2A). The last rise is suspect as a cell cycle pattern because it is in part due to the continued rise in the synchronous culture acceleration while the control has entered the plateau. The initial peak is interesting because it occurs during the lag phase in acceleration. It suggests that there is a cell cycle pattern that is still present and detectable even though whatever causes the lag (perhaps the temperature shift) has produced an overall depression of the acceleration. The general conclusion is that there are one and perhaps two cycles of acceleration in the blocked mutant that are similar to those in unblocked cells.

The middle period is the most reliable part of the curves in which to find cell cycle patterns and we have extended this period by using the double mutant *cdc2.33 wee1.6*. The cells are about half the size of wild-type cells and they show the acceleration plateau about 100 min later. The difference curve in Fig. 4A shows two full cell-cycle oscillations within the middle period and traces of a similar pattern before and after. An important point, which will be discussed later, is that the oscillations show a substantial phase difference compared to those with the single mutant in Fig. 3A. Other points are that the lag phase is much less conspicuous than in the single mutant and that the degree of synchrony is less (Fig. 4F). Most *wee* mutants tend to give poorer synchrony after selection. The number curve for the asynchronous culture (Fig. 4D) continues to increase for a longer period than in the single mutant because the transition point is earlier in the cycle (Fantès, 1983).

The results from a number of experiments are summarized in the cell cycle maps in Fig. 5. These differ from conventional cycle maps in having time along the axis rather than stage in the cell cycle. The top map gives the results from seven experiments with WT cells similar to the ones shown in Figs 1 and 2. The average position of the acceleration steps is exactly at the end of the cycle. This is also very nearly the mid-point of the *S* period at 0.97 of the cycle (Mitchison & Creanor, 1971). The next four maps show the steps with wild-type cells under different conditions of temperature and medium. The important point is that the steps keep the same relative cell cycle timing even when the cycles are shortened in a rich medium (with added yeast extract) or lengthened by poor medium (aspartate as the N source) or by lower temperature. The tight coupling between the steps and division is also maintained after a shift-up in temperature, which delays the first division in minimal medium (Nurse, 1975) but not in a rich medium (Thuriaux *et al.* 1978).

The measurements on *wee* mutants were done in order to test two hypotheses:

(1) That the CO<sub>2</sub> steps occur when cells reach a critical size or mass. This type of control operates on mitosis and DNA synthesis (Nurse & Fantes, 1981), on the rate of mRNA synthesis (Fraser & Nurse, 1978) and on growth in length (Mitchison & Nurse, 1985). The protein content (pg cell<sup>-1</sup>) at the start of the cycle is 8.9 in WT, 6.2 in *wee1.302* and 5.4 in *wee1.6* (Elliott, 1983*b*; Thuriaux *et al.* 1978). The CO<sub>2</sub> step occurs at the start of the cycle in WT where the protein content is 8.9. If this mass value is critical in controlling the step, it will be reached at 0.5 of the cycle in *wee1.302* and at 0.65 of the cycle in *wee1.6*, and the steps should occur at these points.

(2) That the CO<sub>2</sub> steps are associated with the S period. It appears that cells need to reach a critical size before DNA synthesis is initiated and this produces a delay in the timing of the S period in mutants that are fully defective in the *wee1*<sup>+</sup> function. In the case of *wee1.6*, this delay is about 0.38 of the cycle (Dickinson, 1983;

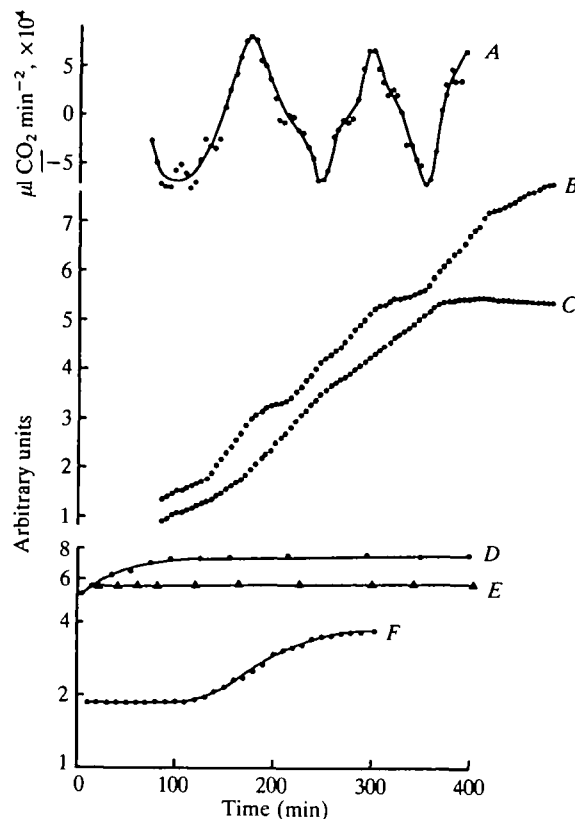


Fig. 4. Acceleration of CO<sub>2</sub> production in a synchronous and an asynchronous culture of the mutant *cdc2.33 wee1.6* in EMM3 shifted up at time zero from 28°C to 36.5°C. Curve A, difference between acceleration curves (B and C). Curve B, acceleration in synchronous culture; value in  $\mu\text{l CO}_2 \text{ min}^{-2} = (\text{a.u.} - 1.0) \times 2 \times 10^{-3}$ . Curve C, acceleration in asynchronous culture; value in  $\mu\text{l CO}_2 \text{ min}^{-2} = (\text{a.u.} \times 2 \times 10^{-3})$ . Curve D, cell number in asynchronous culture; 1 a.u. =  $3.3 \times 10^5$  cells ml<sup>-1</sup>. Curve E, cell number in synchronous culture; 1 a.u. =  $3.3 \times 10^5$  cells ml<sup>-1</sup>. Curve F, cell number in synchronous culture kept at 28°C; 1 a.u. =  $10^6$  cells ml<sup>-1</sup>.



Nasmyth *et al.* 1979). Dr J. Creanor (personal communication, data not shown) has determined the timing of the *S* phase in the partially defective *wee1.302* using pulse-labelling in synchronous cultures (Nasmyth *et al.* 1979) and found the mid-point of the *S* period to be at 0.98 of the cycle, which is not significantly different from WT.

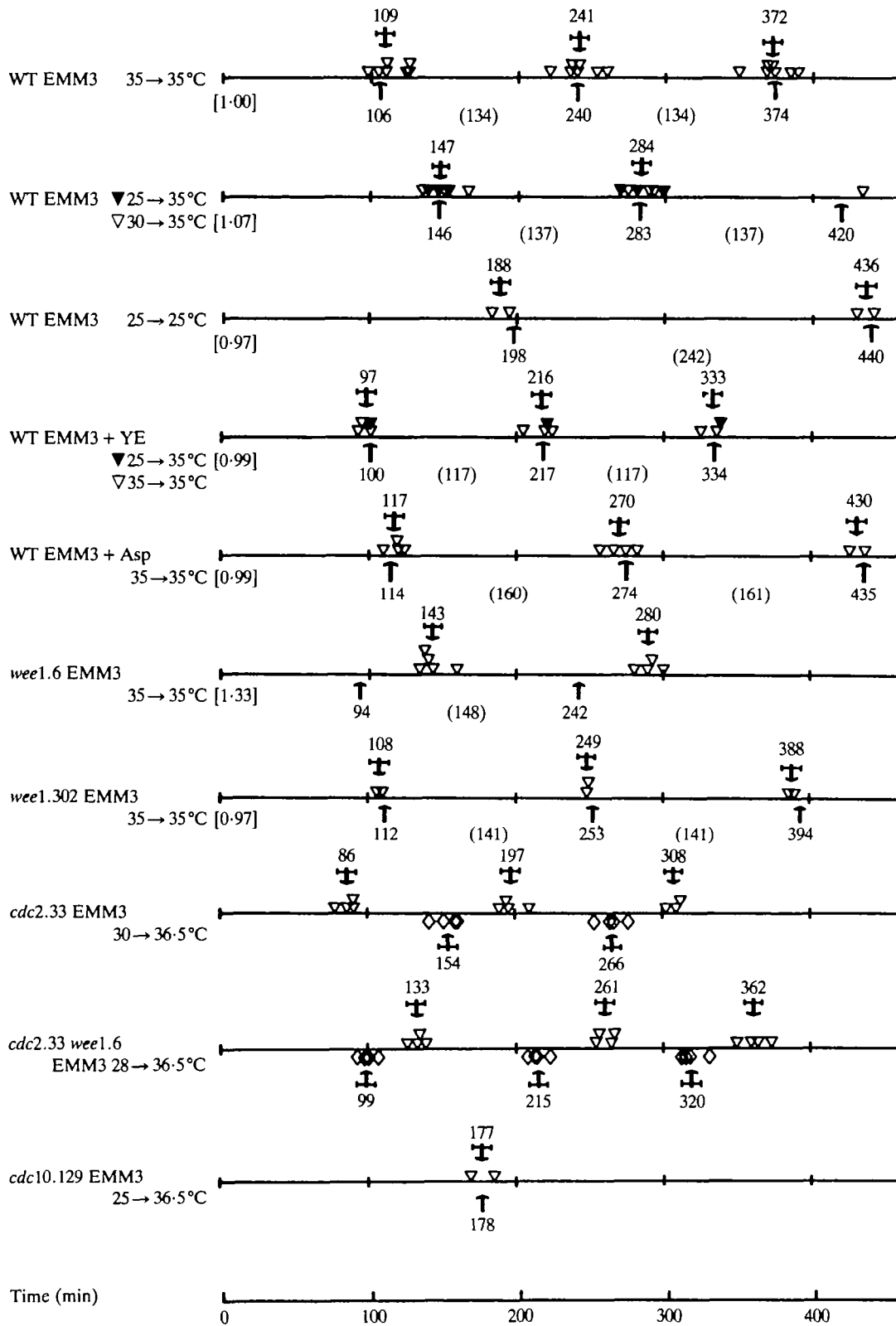
It is clear from the cell cycle maps that the position of the steps supports the second hypothesis and not the first one. There is a delay of 0.33 of the cycle in *wee1.6* and none in *wee1.302*. However, the results with *wee1.6* have to be treated with some caution. The degree of synchrony was lower than in other strains and the cell numbers under-doubled at each step, probably because of diploidization, which is high in this mutant (Thuriaux *et al.* 1978). In addition, there were more marked perturbations in the CO<sub>2</sub> rate curves in the initial stages and in some cases the segments from the synchronous cultures were not lines but curves that were concave downwards.

Two of the cell cycle maps in Fig. 5 refer to the situations in blocked mutants (*cdc2.33* and *cdc2.33 wee1.6*), which are illustrated in Figs 3, 4. The upper triangles show the mid-points of the steps, as in the other maps, but the lower diamonds show the troughs in the difference curves. The average phase difference between the single and the double mutant is 57 min, which is 0.43 of the WT cycle time of 134 min. Another interesting point is that the timing between the steps is about 15% less than in WT. This is shown in Table 1, though the averaging process for these values conceals the fact that in the only case where there is a reliable timing estimate for a second cycle (trough to trough in the double mutant), this is 105 min (22% less than WT). This acceleration is not due to an increased growth rate since Table 1 shows that the rate of protein synthesis does not increase after the *cdc* block and, if anything, decreases.

The last cell cycle map shows the effect of a mutant block imposed with *cdc10.129*. In this mutant, DNA synthesis stops rapidly after the shift-up in temperature but there is one residual division after about 3 h. It is clear that the first CO<sub>2</sub> step kept its normal relationship to division even though there was no *S* period. Unfortunately, it was impossible to define a reliable second step since the CO<sub>2</sub> acceleration formed a plateau.

Table 2 gives the specific rate and acceleration of CO<sub>2</sub> production in asynchronous cultures. Two main points emerge. First, both rate and acceleration are roughly proportional to protein content. It is not perhaps surprising that an extensive cellular property should increase with increasing cell size. Second, both rate and acceleration are much greater in the *cdc* mutants at the start of the acceleration plateau than in WT. The cells of the single mutant are approximately twice the size of the double mutant at 25°C (Elliott, 1983*b*) and they are much larger than WT cells when they reach the plateau.

Table 3 shows the rate of CO<sub>2</sub> production at the time of the rate change points in blocked synchronous cultures of the two *cdc* mutants. The rates double between each cycle and there is also an interesting coincidence between the single and the double mutant. The rate at the first change point in the single mutant (3.18) is about the same as the rate at the second change point in the double mutant (3.07), and the



same is true for the next change points. The points are at different times after shift-up in the two mutants because of the phase difference described earlier. The nature of the control of these rate doublings is unknown but it cannot be doublings in protein content, which take appreciably longer after shift-up than the rate doublings (Table 1).

#### DISCUSSION

These results are in substantial agreement with the measurements made by Hamburger *et al.* (1977) on synchronous cultures of strain 972 made by selection and by heat shocks. They also measured the CO<sub>2</sub> production of single cells and the resulting clones in diver respirometers, an impressive technical feat. Their results showed a linear pattern in rate with a doubling at or near the time of division. Creanor (1978*a*) found the same pattern in complex medium but in minimal medium the pattern was different and appeared to be a stepwise increase in rate. We cannot account for this difference satisfactorily but the experiments were done in a different way, with a different strain of yeast in different medium and with a different method of synchronization. It is possible that there was more perturbation than was suspected during the first 90 min and it is sometimes difficult to distinguish on a semi-logarithmic plot between a partially smoothed-out step and a pattern that is linear on Cartesian coordinates.

In contrast to O<sub>2</sub> consumption, there is comparatively little information about CO<sub>2</sub> production in other systems. Cell cycle fluctuations have been found in early embryos (Frydenberg & Zeuthen, 1960; Zeuthen & Hamburger, 1972). They have also been found in *Saccharomyces cerevisiae* by von Meyenberg (1969) though they are presented in a form that makes it difficult to compare them with our results.

CO<sub>2</sub> production in *S. pombe* is a measure of the glycolytic flux and about half the total ATP production (Hamburger *et al.* 1977). We can ask the question whether it is unusual for a growth pattern in this yeast to show cell cycle periodicities. The answer is no. All growth patterns that have been measured carefully show such periodicities and there is no case of smooth exponential increase. This is true of total dry mass (Mitchison, 1957), cell length (Mitchison & Nurse, 1985), O<sub>2</sub> consumption (Creanor, 1978*b*), total protein (Creanor & Mitchison, 1982), ribosomal RNA (rRNA) (Elliott, 1983*a*), messenger RNA (Fraser & Nurse, 1978), DNA (Mitchison & Creanor, 1971), total acid-soluble pool (Mitchison & Cummins, 1964), the activity of three enzymes (Mitchison & Creanor, 1969), the activity of nucleoside diphosphokinase (Creanor & Mitchison, 1986) and enzyme potential for two

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Fig. 5. Cell cycle maps. Each triangle shows the time of a rate change point in CO<sub>2</sub> production in a synchronous culture estimated from the mid-point of the step in acceleration. The upper arrows and cross-bars give the means and standard errors. The lower arrows give the mean of the mid-points of the steps in cell numbers, i.e. the start of the cell cycle. With the blocked mutants (*cdc2* and *cdc2 wee1.6*), the rate change points are estimated from the mid-rise points of the difference curves and the lower diamonds show the troughs in the difference curves. The values in parentheses are the cycle times and the values in square brackets are the positions of the rate change points in fractions of the cycle.

Table 1. *Timing of rate changes in CO<sub>2</sub> production and doubling time in rate of protein synthesis in synchronous cultures of WT cells and blocked cdc mutants (in EMM3)*

	Mean time between successive mid-points of rises and between successive troughs in the difference curves (min)*	Rate of protein synthesis (doubling time in min)†
972 (WT) (35°C)	134	131 (3:2.9)
<i>cdc2.33</i> (36.5°C)	112	143 (5:5.6)
<i>cdc2.33 wee1.6</i> (36.5°C)	116	137 (4:10.7)

\* S.E. shown in Fig. 5.  
† Mean (*n*: S.E.).

enzymes (Benitez *et al.* 1980). Although the exact details vary, the predominant pattern in 7 out of the 11 cases is one where there is a sharp increase in rate between 0.8 of the cycle and 0.3 of the next cycle. O<sub>2</sub> consumption shows two rate changes per cycle and the total pool does not show sharp rate changes (within the limits of measurement). Our results show that CO<sub>2</sub> production follows the predominant pattern with a rate change at the end of the cycle.

Cell size is an important element in the control both of mitosis and DNA synthesis (Nurse & Fantes, 1981) and the existence of the size mutants that have been isolated during the last decade makes it possible to test whether other periodic events of growth are also size-related. This has only been done in a limited number of cases but it is clear that the rate change points for total protein and rRNA are not related either to size or to the *S* period. They seem instead to be associated with mitosis. On the other hand, cell length and mRNA do seem to be size-related since there is a sharp increase in rate when the cell reaches a critical size. There is also some dependence of enzyme potential on size. There is at present no model to account for these differences.

Table 2. *Specific rate and acceleration of CO<sub>2</sub> production*

Strain	CO <sub>2</sub>		Protein/cell* (pg)	CO <sub>2</sub> rate/ protein	CO <sub>2</sub> acceleration/ protein
	CO <sub>2</sub> rate/cell (μl CO <sub>2</sub> min <sup>-1</sup> , × 10 <sup>7</sup> )	acceleration/cell (μl CO <sub>2</sub> min <sup>-2</sup> , × 10 <sup>9</sup> )			
972 (WT)	2.53	1.74	12.3	0.206	0.141
<i>wee1.302</i>	1.55	1.05	8.6	0.180	0.122
<i>wee1.6</i>	1.27	0.86	6.9	0.184	0.125
<i>cdc2.33</i>	12.76	7.3	—	—	—
<i>cdc2.33 wee1.6</i>	7.32	3.6	—	—	—

Average values in asynchronous cultures in EMM3. Temperature: 35°C for first three strains, and 36.5°C for the last two blocked mutants where values are given at the start of the acceleration plateau (thereafter, the rate increases but the acceleration does not).

\* From Elliott (1983b).

Table 3. Specific rates of CO<sub>2</sub> production at rate change points in synchronous blocked cultures of *cdc* mutants (36.5°C in EMM3)

	Rate change point	Time after shift-up (min)	CO <sub>2</sub> rate/cell ( $\mu\text{l CO}_2 \text{ min}^{-1}, \times 10^7$ )
<i>cdc2.33</i>	1	80	3.18
	2	200	6.30
	3	310	12.82
<i>cdc2.33 wee1.6</i>	1	140	1.56
	2	260	3.07
	3	370	6.47

Rate changes in CO<sub>2</sub> production are tightly coupled in WT cells to division and the *S* period (which are contemporaneous) and they are not size-related, as is shown most obviously with *wee1.302*. The *S* period appears to have priority in this coupling since when it moves in *wee1.6*, the rate change in CO<sub>2</sub> production also moves by the same amount. But if there is no *S* period, as in the first division of *cdc10* after a shift-up, then the rate change maintains its close association with division.

Association with the *S* period is a novel feature in comparison with other periodic events in growth in *S. pombe* and it is worth emphasizing that it depends entirely on the results with *wee1.6*, which were not altogether satisfactory, as we have pointed out earlier. If these results were discounted, the patterns of association would be like those for protein and rRNA. Assuming, however, that the results are correct, a very simple model would be that a rate-limiting component of the glycolytic pathway, perhaps an enzyme, doubled its rate of synthesis (or activity increase) at the time of the *S* period. If it followed a linear pattern like the other enzymes assayed by Mitchison & Creanor (1969), and the rate of production of CO<sub>2</sub> was proportional to the amount of the enzyme, then this rate would show the linear pattern of increase that we have found. It is possible that this could be a gene-dosage effect where doubling the number of gene copies would double the rate of production of the enzyme. This suggestion, however, can only apply to the normal cell cycle since the rate changes persist after a block to DNA synthesis. In addition, this aspect of the control could only be a fine tuning during the cell cycle since the overall level of CO<sub>2</sub> production increases with cell size both after a block and in normal growth when WT cells are compared with *wee* cells.

The most striking aspect of our results is the unequivocal demonstration that the periodic pattern in the rate of CO<sub>2</sub> production, an extensive property of the cell, persists for up to two cycle times after a mutant block to the DNA-division cycle. The survival of cell cycle events after an inhibitor block or after enucleation is by now a well-known phenomenon in eggs and early embryos. Surface contraction waves persist in amphibian eggs (Yoneda *et al.* 1982; Hara *et al.* 1980) and in sea-urchin eggs (Yoneda *et al.* 1978). So also do cycles of maturation promoting factor (MPF) in amphibian eggs (Gerhart *et al.* 1984). Since contraction waves continue in enucleate egg fragments, Hara *et al.* have described the control as a 'cytoplasmic clock'. Eggs, however, may have somewhat different cell cycle controls since the

cycles are short and invariant and cannot be controlled by cell size in a non-growing system.

There is much less information about persisting periodicities in growing systems. The earlier evidence has been summarized by Mitchison (1971). More recently, Aerts *et al.* (1985) found that cycles in the cytoplasmic pH of *Dictyostelium* continued after a hydroxyurea block, and Edwards & McCann (1983) showed that stepwise rises in O<sub>2</sub> uptake in the prokaryote *Alcaligenes* persisted after a block by naladixic acid. However, most of the directly relevant results come from earlier work on *S. pombe*. Length growth in single cells of *cdc2.33* shows a single rate change at about 170 min after a shift-up, but not in all cells (Mitchison & Nurse, 1985). Apart from CO<sub>2</sub> production, the best evidence for surviving periodicity comes from the steps in nucleoside diphosphokinase (Creanor & Mitchison, 1986), and arginase and sucrase potential, which persist for up to two cycles after a mutant block (Benitez *et al.* 1980). However, enzyme potential, which is the maximum rate of increase in activity induced in a sample taken from a synchronous culture, is a somewhat indirect way of following cell cycle events, especially since it does not appear to be controlled by gene dosage in the normal cycle. There is a different situation with total protein (Creanor & Mitchison, 1984) and rRNA (Elliott, 1983*a,b*). Although both these cell components continue to rise at an increasing rate after a mutant block, the cell cycle periodicities in rate disappear. It is puzzling that one extensive property of the cell, CO<sub>2</sub> production, should show continued cell cycle periodicities in rate after a mutant block whereas another one, protein synthesis, does not.

There are two interesting aspects of the timing of the CO<sub>2</sub> rate changes after a block. The first is that the intervals are shorter than the normal cell cycle by 15–20%. There was a similar, though less-marked, shortening (5–10%) in sucrase potential (Benitez *et al.* 1980, fig. 20), but not in nucleoside diphosphokinase activity (Creanor & Mitchison, 1986). The second point is the phase difference of 0.43 of the cycle between the rate changes in *cdc2.33* and in *cdc2.33 wee1.6*. The most likely explanation is that these mutants would have approximately the same phase difference of 0.33 of the cycle at the permissive temperature as *wee1.6* and WT at 35°C, and that this difference is maintained after the shift-up.

Unlike the situation in eggs, it is only possible to follow rate periodicities in blocked cells of *S. pombe* for one or two cycle times. Thereafter the rate or the acceleration reaches a constant plateau value and the periodicities become undetectable. This happens with CO<sub>2</sub> production, enzyme potential, and protein and rRNA synthesis. There is evidence from protein synthesis and from potential that the plateau occurs when the enlarging cells reach a critical ratio of protein/DNA. Hence *wee* cells take longer to reach the plateau and there is greater opportunity to measure the periodicities. But the situation is not altogether simple since rRNA synthesis reaches a plateau well before protein synthesis and the critical ratio for sucrase potential differs from that for arginase potential. With CO<sub>2</sub> production, the *cdc2.33 wee1.6* cells reach a plateau later than the *cdc2.33* cells but they have lower values of rate and acceleration.

The most intriguing question raised by these results is the nature of the oscillator that controls CO<sub>2</sub> production after a block to the DNA-division cycle. A minimum assumption is that it is a control specific to this cell property and that it is normally entrained by an event in the DNA-division cycle, probably the *S* period. It is free-running after a block with a timing that is appreciably less ( $\approx 15\%$ ) than the normal cycle time, but it cannot be detected once the acceleration plateau has been reached. However, enzyme potential, nucleoside diphosphokinase activity and perhaps cell-length growth also show persistent rate changes after a block, and it is possible that all three properties are controlled by a single master oscillator.

Further work is obviously needed to track down the number and nature of the oscillators and their relations to the DNA-division cycle. They are not restricted to fission yeast since there is overwhelming evidence of their existence in eggs and some evidence in other cell types. But if they manifest themselves in rate changes, it needs precise and careful techniques to detect them. The clear demonstration of the rate changes in this paper required the calculation of second differentials and simultaneous measurements on synchronous and asynchronous cultures prepared from the same starting culture.

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