LINEAR SYNTHESIS OF SUCRASE AND PHOSPHATASES DURING THE CELL CYCLE OF SCHIZOSACCHAROMYCES POMBE

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SUMMARY

The synthesis of sucrase, acid phosphatase and alkaline phosphatase has been followed in synchronous cultures of the fission yeast Schizosaccharomyces pombe prepared by gradient sedimentation. These three enzymes follow a linear pattern of synthesis through the cell cycle, with a doubling in rate at a 'critical point' about one-fifth of the way through the cycle.

Sucrase can be rapidly derepressed by lowering the glucose concentration in the medium. This has been used to measure the sucrase 'potential' or capacity to synthesize sucrase on derepression. The potential exists at all times in the cycle, and follows a stepwise pattern with a sharp rise at the critical point.

These results suggest that the functional genome doubles at the critical point. Since, however, the period of DNA synthesis is nearly one-third of a cycle before this point, there must be an appreciable delay between chemical replication and functional replication of the genome. In this respect S. pombe, a eukaryotic cell, differs markedly from bacteria.

Other physiological events take place near the critical point, and a tentative model is suggested of what may be happening at the chromosomal level.

Experiments with cycloheximide indicate that there is a delay between the synthesis and the appearance of the active enzyme in the case of sucrase and alkaline phosphatase.

INTRODUCTION

The patterns of enzyme synthesis in synchronous cultures have been one of the most interesting aspects of cell cycle work in the last few years, both because of the light they throw on regulation and because they emphasize that there is an ordered sequence of chemical changes throughout the cycle (for recent short reviews, see Donachie & Masters, 1969; and Mitchison, 1969a). In many cases the total protein of a cell is synthesized continuously and follows a roughly exponential curve through the cycle; but individual proteins, represented by enzymes, seldom follow this pattern. The majority of enzymes are made discontinuously for a limited period of the cycle, like DNA in higher cells, and the timing of this synthesis period or step varies from enzyme to enzyme. Another, less-frequent pattern is shown with alkaline phosphatase and β-galactosidase in Escherichia coli (Kuempel, Masters & Pardee, 1965) and with alkaline phosphatase in Bacillus subtilis (Donachie, 1965). These enzymes, when repressed, are made continuously, but instead of following an exponential curve of synthesis they follow a linear pattern. The rate of synthesis remains constant for a generation time...
and then doubles sharply at a characteristic point in the cycle. In a synchronous culture covering three generations, this gives a pattern of three straight lines with slopes of 1, 2 and 4 and with the two rate-change points at the same times in two successive cycles. It was suggested that this might be a gene dosage effect, with the rate of synthesis doubling when the appropriate structural gene doubled. This was supported by the fact that the ‘potential’ for these enzymes in *E. coli* also doubles at the same points—the potential here being the rate of synthesis in samples removed from the synchronous culture and induced or derepressed.

*Schizosaccharomyces pombe*, a fission yeast and a eukaryote, shows both continuous and periodic enzyme synthesis. Five ‘step’ enzymes have so far been found which show periodic synthesis: aspartate transcarbamylase and ornithine transcarbamylase (Bostock, Donachie, Masters & Mitchison, 1966); tryptophan synthetase, alcohol dehydrogenase and homoserine dehydrogenase (A. A. Robinson, unpublished). Four other enzymes are synthesized continuously: maltase, sucrase, alkaline phosphatase and acid phosphatase, and it is with the last three of these that this paper is concerned. Maltase has not been analysed in detail because it does not follow an exponential curve of synthesis in control asynchronous cultures (Bostock et al. 1966).

This work started with two main objectives. One was to see whether or not the continuous synthesis of the 3 enzymes showed the linear pattern which had been described for bacteria, though only in brief reports. This pattern has now been shown to exist in this yeast. The second objective was to see where were the rate-change points and potential doublings in a eukaryotic cell cycle with a very limited period of DNA synthesis (*S*-period). The original prediction was that these points would be coincident for the three enzymes and would fall in the *S*-period. The results show, however, that although the points are coincident they fall not in the *S*-period but nearly a third of a cycle later. It seems therefore that there is a considerable delay between the chemical replication of the genome and its ‘functional’ replication.

**METHODS**

Synchronous cultures of *Schizosaccharomyces pombe* (N.C.Y.C. 132) were prepared by the gradient sedimentation method of Mitchison & Vincent (1965). In a typical experiment, an asynchronous exponential-phase culture of 4 l. grown at 32 °C was harvested when it reached a concentration of 2—4 × 10⁶ cells/ml. The cells were collected by filtration on a Whatman no. 50 paper, made up to 5 ml, and then layered on to the top of an 80-ml linear gradient of 10—40 % glucose in medium in a 100-ml centrifuge tube. After centrifuging for 5 min at 500g the cell layer spread out and moved two-thirds of the way down the tube. About 5 ml of the top layer of small cells were removed by a syringe and added to 150 ml of fresh medium at 32 °C to give a concentration of 1.5 × 10⁶ cells/ml. This synchronous culture was then followed for 6 h, with 1-ml duplicate samples taken every 5 min for enzyme assay. The cells from each sample were collected on a membrane filter (Oxoid or Millipore Microweb or Polyvic) which was then washed several times with distilled water, inserted into a disposable polystyrene tube and freeze-dried over P₂O₅. Samples were also taken every 15 min for subsequent staining (Mitchison, 1969b) and determination of the cell plate index (percentage of cells showing cell plates).

Cell numbers were not normally followed but examples of synchronous cultures in which both cell number and cell plate index were followed are given in Bostock et al. (1966) and Mitchison (1969b). The doubling in cell number was a little later than the peak of the cell plate index. On average, this delay was 11 min or 0.075 of a cell cycle (where 1.0 is the whole...
Enzyme synthesis during the cell cycle

The degree of synchrony was not perfect and the first doubling in numbers took place over about 1 h (0.4 of a cycle).

The 6 h for which the synchronous cultures for enzyme assay were followed covered about half of the first cycle, all the second cycle and most of the third cycle. There was some variation in the time of appearance of the first cell plate peak which was due to differences in the length of time after cell division during which the two daughter cells remained adherent. The top layer in the gradient contained the smallest single cells, whereas cell pairs sedimented with the lower layers. If the daughter cells separated soon after division there was nearly a complete cell cycle before the first cell plate peak in a synchronous culture. But if they remained adherent as cell pairs for half a cycle after division, then there was only half a cycle in the synchronous culture before the first peak (Fig. 6).

The medium used in the sucrase and alkaline phosphatase experiments was E.M.M. (1), a minimal medium with glucose as the carbon and energy source and ammonium chloride as the nitrogen source (Mitchison & Gross, 1965; Mitchison, 1969b). This is a low-phosphate medium and was unsuitable for measuring basal levels of acid phosphatase which would have been derepressed by the absence of phosphate in the medium in the later stages of exponential growth. The phosphate concentration was therefore raised from 10 mg/l. to either 100 or 300 mg/l. for the acid phosphatase experiments. With E.M.M. (1) the limiting component of the medium is phosphate, while with E.M.M. (1) plus either of the phosphate supplements it is glucose. The synchronous culture runs were always completed before the end of the exponential phase of growth.

Asynchronous control cultures were made in exactly the same way as the synchronous cultures except that the whole contents of the gradient tube were shaken up and mixed together after centrifuging. Enough of the resulting cell suspension was then added to fresh medium to give a culture of the same volume and cell concentration as the synchronous cultures. These control cultures were asynchronous as judged by the most sensitive test available, the cell plate index. This index remained at about 10 % and did not show the systematic waves which were characteristic of the synchronous cultures.

The enzyme assays were done on the frozen-dried cells in the plastic tubes. The substrate mixture for sucrase was 1 vol. 5% (w/v) sucrose (Kerfoot) + 2 vol. 0.067 M phosphate (Serensen) buffer, pH 6.8, in 0.01 M ethylenediaminetetra-acetate (Na salt). One ml of this mixture was added to the cells and incubated at 32 °C. After incubation (several hours for basal level sucrase), the resulting glucose was measured by adding 0.5 ml Glucostat Special reagent (Worthington), incubating for a further period (usually 30 min) and then stopping the reaction with 1 ml 0.25 N HC1. The substrate mixture for alkaline phosphatase was 1 vol. 0.1 M p-nitrophenyl disodium orthophosphate (B.D.H.) + 1 vol. M tris(hydroxymethyl)aminomethane buffer, pH 8.0, +8 vol. water; 1 ml of this mixture was added to the cells, incubated at 32 °C and the reaction stopped with 1.5 ml of 0.5 M Na,HPO, in 0.5 N NaOH. The acid phosphatase assay was the same except that the substrate mixture was 1 vol. 0.1 M nitrophenyl phosphate + 4 vol. M acetate buffer pH 3.0 + 5 vol. water, and the incubation temperature was 22 °C. With all three assays, the cells were centrifuged down after the reaction had been stopped, and the supernatant decanted off and measured in absorbance at 400 nm in a spectrophotometer against a blank without cells. All the samples from a synchronous culture were incubated for the same length of time. Tests with all three enzymes showed that there was a linear relation between absorbance and quantity of cells. The membrane filters were left in the tubes during the assays except during the incubation with the Glucostat reagent. Absolute values of enzyme activity are given in Fig. 6.

OBSERVATIONS

Properties of the enzymes

The pH response of the three enzymes is shown in Fig. 1. The optimum pH for acid phosphatase was 3.0 but there was little change in activity between pH 2.5 and 4.0. The response curves of sucrase and alkaline phosphatase had sharper peaks at pH 6.6 and 8.0.
The Michaelis constants ($K_m$), determined from the reciprocal plots in Fig. 2, were 9.7 mM for sucrase, 9.4 mM for alkaline phosphatase and 6.7 mM for acid phosphatase. When sucrase was derepressed by dilution (see below) the $K_m$ fell to 2.2 mM. This suggests that sucrase activity is due to two or more iso-enzymes with different $K_m$ and that their relative proportions change on derepression. Sucrase (invertase) iso-enzymes are known in budding yeast, although the $K_m$ is the same for external and internal sucrase (Gascón, Neumann & Lampen, 1968).

Some enzymes in budding yeast are known to be external to the cell membrane but within the cell wall. This possibility was explored by comparing the activity of washed living cells and of the same number of frozen-dried cells. The acid phosphatase activity was the same, indicating that this enzyme is external, as it is in budding yeast (Schmidt et al. 1963; McLellan & Lampen, 1963; Weimberg & Orton, 1964). With alkaline phosphatase the activity of living cells was 6% of that of frozen-dried cells, showing that this enzyme is largely internal, which again is similar to the situation in budding yeast (McLellan & Lampen, 1963). With sucrase, the activity of living cells was 70% of that of frozen-dried cells (80% for derepressed sucrase). This suggests a combination of an external and an internal enzyme, as in budding yeast (Sutton & Lampen, 1962; Gascón et al. 1968). Only a small proportion of the enzymes in frozen-dried cells was soluble after 30 min at 0 °C in the buffers used for assay (8.6% for sucrase, 3.7% for acid phosphatase and 0.4% for alkaline phosphatase).

The state of repression of the enzymes was investigated because we wished to measure the rate of synthesis of enzyme during the derepression of samples taken from a synchronous culture. For this purpose it was necessary that the derepression should be rapid. In other yeasts, alkaline phosphatase is constitutive (Weimberg & Orton, 1963), acid phosphatase can be derepressed by reducing the phosphate in the medium (Weimberg & Orton, 1963; Schmidt et al. 1963) and sucrase can be derepressed by reducing the glucose in the medium (Dodyk & Rothstein, 1964; Gascón & Lampen, 1968). The same situation was found in S. pombe. The level of alkaline phosphatase could not be changed by an alteration of the phosphate level in the medium. The acid
phosphatase activity, on the other hand, increased by a factor of \( \times 8 \) if a growing culture was transferred to a medium without phosphate. This derepression was too slow to use in a synchronous culture because there was a lag of at least 1 h before the enzyme level rose, presumably because of the large internal phosphate pool which had to be depleted. Also the growth rate of the cells started to fall off as the enzyme level rose. An interesting point about this derepression was that it could be stopped within 10 min by adding phosphate back to the medium (J. W. May, unpublished results). If the repression is acting at the genetic level, this suggests that the messenger RNA is unstable. The acid phosphatase level also remained constant for at least 100 min after the addition of the phosphate, indicating that the enzyme was stable.

Sucrase activity was found to be dependent on the glucose concentration in the medium (Fig. 3). The activity was fully repressed at 1 % glucose (the concentration in the minimal medium) and rose at both lower and higher concentrations. The derepression procedure that was used with the synchronous cultures was to dilute the medium 20 times with medium made up without glucose (so reducing the glucose from 1 % to 0.05 % but leaving the other components unchanged) and then collect samples of 20 times the normal size. The kinetics of this derepression are shown in Fig. 4. The activity started to rise after 15 min and followed a linear course from 20 to 100 min. During this time the growth of the cells proceeded normally as judged by the increase in optical density. There was no change in activity in cells transferred to medium without glucose, showing that the enzyme was stable in the absence of an energy source. Sucrase activity could also be increased by transferring cells to a medium which contained sucrose rather than glucose. There was, however, a lag of about 1 h before the enzyme level started to rise and during this period the cells stopped growing. This lag may be due to the inability of the cells to take up sucrose so that there is a delay until the external sucrase has produced enough glucose to allow growth to resume (Islam & Lampen, 1962).
In common with other workers in the field of synchronous cultures, we have not shown that a rise in activity is directly due to net synthesis of the enzyme protein in question. We have, however, used cycloheximide to investigate the relation of activity to synthesis. This antibiotic is an inhibitor of protein synthesis in many eukaryotes, and is thought to act at the ribosomal level by preventing the transfer of amino acids from the s-RNA to the growing polypeptide chain (Siegel & Sisler, 1965). Table 1

![Fig. 4. Kinetics of sucrase derepression. A, growing culture diluted × 20 with medium without glucose at time 0. B, same culture undiluted. C, same culture transferred to medium without glucose. Sample size in A is × 20 that in B and C.](image)

Table 1. Rate of incorporation of leucine after inhibition by cycloheximide (CH)

(One-ml samples from exponential culture (4 × 10⁶ cells/ml at time 0) incubated at 32 °C for 10 min with 2 μCi of L-[U-¹⁴C]leucine (Amersham, specific activity 10 mCi/mM). Acid-insoluble radioactivity (Mitchison & Gross, 1965) measured in a low-background Geiger counter. The first (control) pulse was made just before the addition of CH.)

<table>
<thead>
<tr>
<th>Time of pulse label after addition of CH (min)</th>
<th>20 μg CH/ml</th>
<th>100 μg CH/ml</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Counts/s</td>
<td>Activity as % of control</td>
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<tr>
<td>Control (−10 to 0)</td>
<td>25.8</td>
<td>—</td>
</tr>
<tr>
<td>5−15</td>
<td>3.6</td>
<td>14</td>
</tr>
<tr>
<td>10−20</td>
<td>3.3</td>
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</tr>
<tr>
<td>60−70</td>
<td>1.0</td>
<td>4</td>
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</table>
Enzyme synthesis during the cell cycle shows that it is effective in *S. pombe*, and at 100 μg/ml reduced leucine incorporation to 8% after about 10 min. But cell growth continues for some time: the rate of increase of optical density is still 40% of its original value after 2 h with cycloheximide at 100 μg/ml. Figure 5 shows the effect of cycloheximide on the rise in activity of the three enzymes in an asynchronous culture and on sucrase derepression. The increase in acid phosphatase activity is stopped at once by the inhibitor, but there is an appreciable delay before this happens with the other two enzymes. This delay is about 30 min (0.21 of a cycle) with alkaline phosphatase and about 15 min (0.1 of a cycle) with sucrase both at basal level and during derepression. A delay of 1 h was found by Fukuhara (1966) in a similar experiment on the inhibition of synthesis of iso-2-cytochrome C by cycloheximide in budding yeast and was interpreted as being caused by a lag between the synthesis of a precursor and its conversion into the final form (see Bilezikian, Kaempfer & Magasanik (1967) for a similar bacterial situation). We interpret our results in the same way and conclude that there is a 'precursor delay' between the synthesis of these two enzymes and their activation, which might be due to assembly of subunits or conformational changes or the temporary lack of a suitable site. Thus the enzyme synthesis which is responsible for an activity change will precede that change by a time interval equal to the precursor delay. As will be seen later, this affects the timing of enzyme rate changes and of potential changes in the cycle. It provides a simple explanation of the delay of 15 min in the derepression of sucrase (Fig. 4), since this is equal to the precursor delay. A final point from the cycloheximide results is that the absence of any large decreases in activity shows that the enzymes are relatively stable over periods of a few hours.
Enzyme synthesis in synchronous cultures

Each synchronous culture was assayed for one enzyme only on samples (sometimes in duplicate) taken every 2-5 or 5 min. Three results are shown in Fig. 6 and it can be seen that they appear to show a linear pattern of synthesis, with a doubling (approximately) at a stage in the cycle which is later than the cell plate peak. Figure 6 also shows that it is difficult to decide by visual inspection whether curves of this kind are made up of linear segments unless the assays are very accurate. This is not surprising since the maximum difference between an exponential curve which doubles its rate and the line of best fit is less than 3%.

The results were therefore analysed with a statistical method designed by D. A. Williams and set out in the Appendix. In essence, this method determines whether
the results give a better fit to a smooth curve or a set of linear segments. Two restrictions are imposed: that there should be only three segments (since there were three cell cycles in each experiment), and that the two rate-change points should not be less than 90 min apart (0.62 of a cycle). There is no other restriction on the linear model and the method produces the lines of best fit with the corresponding rate-change points. In general these points turned out to be very nearly the same as those determined by inspection. The sucrase data produced straight lines on a semilogarithmic plot, so the smooth curve for comparison with the three lines was taken to be an exponential. The alkaline and acid phosphatase results deviated slightly from an exponential (presumably because of the changing conditions in a batch culture), so

Table 2. Statistical test on synchronous cultures and asynchronous control cultures

(The first three columns are the total number of cultures which gave the best fit to either the linear model or the smooth curve model (exponential or polynomial) or neither. The cultures in the last column had so much variation that the test had no discrimination.)

<table>
<thead>
<tr>
<th></th>
<th>Linear</th>
<th>Smooth curve</th>
<th>Neither</th>
<th>No discrimination</th>
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<td>2</td>
</tr>
<tr>
<td>Control</td>
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<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Synchronous</td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
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<td>1</td>
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<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
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<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
<td>13</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

a polynomial curve was used instead of an exponential. In some cases the statistical verdict was either that the data did not fit either model or that the data were too variable to allow discrimination between the models.

The results from 21 synchronous cultures and 18 asynchronous controls are shown in Table 2. There is good evidence that the synchronous cultures give a better fit to the linear model and that the asynchronous control cultures give a better fit to the smooth curve. Only one of the analysed synchronous cultures has been excluded. This was a sucrase experiment which showed two rate-change points in the second cycle (one at the beginning and one at the end) and can be regarded as anomalous. The rate-change points from the 16 synchronous cultures that fitted the linear model are plotted on cell-cycle maps in Fig. 7. There is no significant difference between the means of the three enzymes. There is also a large scatter which is characteristic of most of the other cell-cycle maps that have been published (e.g. Bostock et al. 1966; Masters & Pardee, 1965). It remains to be seen whether this scatter is due to experimental errors or to genuine variability between cultures. The points for acid phosphatase in the second cycle are earlier than those in the third cycle, but since this
does not happen with the other enzymes, where if anything the tendency is the other way, we do not attach any particular significance to it. There is no marked correlation in individual experiments between the position of the point in the second cycle and in the third.

With sucrase and alkaline phosphatase, the points where the rate of enzyme synthesis doubles will be earlier in the cycle than the rate-change points because of the precursor delay mentioned earlier. These points of synthesis doubling are shown in Fig. 7.

It may be thought that the changes in rate are surprisingly rapid, considering that it takes about 1 h (0.4 of a cycle) for the cell numbers to double in a synchronous culture step. Drawing lines through model curves, however, shows that the sharpness is largely constructive, and the statistical method will give a linear answer even if only 0.6 of the cycle is linear and the remaining 0.4 is curved.

Three synchronous cultures were made with the glucose concentration in the medium raised from 1% to 10%, and then assayed for sucrase. This caused partial derepression of the sucrase, though not by a large factor (Fig. 3). The pattern of enzyme synthesis was continuous (but it was uncertain whether it was linear) and not in steps, as would be predicted from the 'oscillatory repression' theory for bacteria (Donachie & Masters, 1969).
Sucrase derepression in synchronous cultures

The changes in sucrase potential during the cell cycle were followed by measuring the rate of increase of sucrase activity in samples taken from a synchronous culture and derepressed by diluting the glucose in the medium. Samples were taken every 15 min, diluted 20 times with medium without glucose, and then assayed at 25, 50 and 75 min after dilution when the derepression curve was linear (Fig. 4). The results of an experiment are shown in Fig. 8 with lines drawn for each sample through its three assay points. The slopes of these lines are plotted in Fig. 9A together with the timing of the cell plate peaks. The timings are given with respect to the middle assay at 50 min,

![Graph showing sucrase activity over time](image)

i.e. if a sample was taken 50 min before the cell plate peak in the main synchronous culture, the rate of derepression (or potential) for that sample is taken to be the rate for cells at the cell plate peak. This seems to be the most reasonable assumption since cells in the samples continued to grow at the same rate as those in the main culture, but it should be realized that this is a little arbitrary and leaves some doubt about the precise timing of a change in potential.

The conclusions from Fig. 9A are: (1) sucrase is derepressable at all stages of the cycle; (ii) the sucrase potential remains constant for most of the cycle and rises sharply at a characteristic point; and (iii) these rises are steps which are less than doublings. Fig. 9B shows a comparable result for an asynchronous control. There are no steps but there is the same tendency for the potential to increase at a rate which is less than exponential, presumably because of the changing conditions in a batch culture.
The mid-points of each step (where the potential has reached half its final value) in six synchronous cultures are plotted on a cell cycle map in Fig. 7. The mean is at 0.38, but because of the precursor delay this represents the step in apparent potential. Allowing for the precursor delay of 0.1, the mean position of the true potential step is at 0.28.

![Graph](image)

**Fig. 9.** A, Change of sucrase potential in a synchronous culture. Each point gives the slope of a sample in Fig. 8 at the time of the middle assay (50 min after the time of taking the sample from the culture). Arrows show the position of the cell plate peak. B, Change of sucrase potential in an asynchronous control culture. The method is the same as that for A.

There is no significant difference (F test, $P = 0.05$) between the timing of the potential step (0.28) and of the points where the rate of synthesis doubles (0.18, 0.09, 0.23). We therefore conclude that within the experimental errors the rates of synthesis of all three enzymes and sucrase potential change at what will be called the 'critical point'. Taking an over-all mean, this critical point is at 0.20 of the cycle.

**DISCUSSION**

Changes in potential very similar to those shown for sucrase in Fig. 9A have been found for nine enzymes in *B. subtilis* and *E. coli* (tabulated in Donachie & Masters, 1969; and Mitchison, 1969a). Indeed this pattern with a relatively sharp doubling at a part of the cycle characteristic for each enzyme seems to be the rule in bacteria, apart from an exceptional case in the work of Nishi & Horiuchi (1966) which is discussed by Donachie & Masters (1969). These are good reasons for believing that the potential for an enzyme doubles at the time in the cycle when its structural gene doubles. The most direct evidence is that sucrase potential in synchronous cultures of *B. subtilis* doubles when the sucrase-transforming capacity of the DNA doubles (Masters & Pardee, 1965). A prediction would be that the order and spacing of the potential doubling steps of a series of enzymes during the cycle should be the same as the order and spacing of the corresponding genes on the bacterial chromosome, provided that replication lasted the whole cycle and that there was only one replicating
Enzyme synthesis during the cell cycle

Enzyme synthesis during the cell cycle has been shown to be approximately true for six enzymes in *E. coli* B/r by Donachie & Masters (1969) and more precisely true for three of them by Helmstetter (1968) using an elegant method of preinducing the enzymes before the culture is synchronized. Another prediction is that a block to DNA synthesis should also stop any further increase in potential even if growth continues. This has been shown to happen with α-serine deaminase by Donachie & Masters (1966) and with β-galactosidase by Pato & Glaser (1968). Finally, there is evidence both in *Neurospora* (Donachie, 1964) and in *E. coli* (Pittard & Ramakrishnan, 1964) that there is a gene dosage effect on enzyme levels: the levels rise proportionately to the increase in gene number.

The only work on enzyme potential in eukaryotes is that of Knutsen (1965, 1968) on *Chlorella*. It is difficult to compare his results with ours since the potential curve is periodic and not stepped, and the pattern of synthesis of the enzymes at basal level is different (stepped) in two cases and unknown in the third. We therefore accept the conclusion from prokaryotes and, at this stage of the argument, interpret the sucrase-potential data as indicating that the sucrase genes double at the critical point, about one-fifth of the way through the cycle.

The linear pattern of synthesis of the two basal level enzymes (sucrase and acid phosphatase) and of the constitutive enzyme (alkaline phosphatase) also has parallels in the prokaryotes. In these cases (Kuempel et al. 1965; Donachie, 1965; see Introduction) the rate changes have also been interpreted as gene-dosage effects—the rate of basal synthesis doubling when the appropriate gene doubles. This view is supported by the fact that the rate-change points are coincident with the points of potential doubling with two enzymes in *E. coli*. In parenthesis, it would be valuable to establish firmly this important point of linear synthesis in prokaryotes. The existing reports are brief and do not include controls or statistical analysis. Careful investigation might also show that patterns of enzyme synthesis which have been described as exponential (e.g. fully induced β-galactosidase in *E. coli*; Abbo & Pardee, 1960; Cummings, 1965) are in fact linear.

In this yeast, three different enzymes double their rate of synthesis at the critical point and the potential for one of them also doubles at the same point. The prediction from the prokaryotic evidence would be that the DNA should also double sharply at this point. Here, however, we meet the interesting paradox that although the DNA does double sharply, it does not do so at the critical point.

A word of explanation is needed about the unusual pattern of DNA synthesis in *S. pombe* (Bostock et al. 1966). This yeast differs from most animal cells in having the cell cycle appreciably out of phase with the nuclear cycle (from one nuclear division to the next). If the cell cycle is taken as starting at cell division and running from 0 to 1.0, then nuclear division happens at 0.75, the cell plate first appears at 0.85 and the cell plate peak in synchronous cultures is at 0.925 (Mitchison & Walker, 1959; Mitchison, 1969b). DNA synthesis takes place rapidly (in perhaps 10 min) at about the time of appearance of the cell plate (0.85) after nuclear division and before cell division (Fig. 7). The DNA/nucleus increases from the 1C to the 2C value and then remains at 2C until the next nuclear division. The DNA which is used for most of a cycle has been replicated a little before the start of that cycle and, to use the G1, S, and G2 terminology (as it

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should be, with respect to the nuclear cycle), there is no $G_1$, a short $S$ and a long $G_2$.

For each cycle therefore the critical point is one-third of a cycle (0·35 or 50 min) after the time of DNA synthesis. In other words, there appears to be a substantial delay between chemical replication of the genome and what can be called ‘functional’ replication. This situation is strikingly different from that in bacteria.

The reasons for this delay are less certain. It is unlikely to be a delay between transcription and translation because it is much longer than the 15-min delay in sucrose derepression (Fig. 4). In any case, this 15-min delay can be accounted for by the precursor delay discussed earlier. The rate-change of an enzyme might be due to a pulse of stable message, but this again seems unlikely both because the evidence from the acid phosphatase derepression suggests an unstable message and also because it provides no explanation of the potential doubling. We are inclined to favour an explanation in terms of chromosome changes, but before describing this model, some other events which also happen at or near the critical point must be considered.

Swann (1962), using a single cell method with *S. pombe*, found a period of ‘gene replication’ during the cycle where the genetic target for u.v. damage appeared to double. This period is not, surprisingly, at the time of DNA synthesis but is at a point in the cycle (0·29 after division, using our criterion for the start of the cycle) which is not significantly different from the critical point. He also found a tenfold increase in resistance to u.v. killing during this period and a fall at the end of the cycle. In subsequent unpublished work he has found that the increase in u.v. resistance is associated with a shift in the action spectrum from that of nucleic acid to one with a peak at 280 nm which broadly resembles the absorption of protein. He has also shown that resistance to $^{32}$P decay in a ‘suicide’ experiment (after a prolonged label) is very similar to that for u.v., though the increase in resistance at the critical point is even more marked. During the resistant phase of the cycle after the critical point, a proportion of the cells survived as many as 3000 single-strand breaks in their DNA and produced viable clones. This implies not only an efficient repair mechanism but also some framework to hold the DNA fragments in place during the repair process.

We and Swann suggest that the following model will account for some of these events. After DNA synthesis, only one of the two resulting chromatids would be available for transcription. They would also be closely linked and treated as one target for u.v. damage. These two chromatids would separate within the nucleus at the critical point (in a way which might be analogous to metakinesis in metaphase chromosomes) and provide two u.v. targets. They would also both become available for transcription. If each chromatid acquired a protein backbone at this point, it would explain the shift in the u.v. action spectrum and the increased resistance to $^{32}$P decay. Finally the backbone would be removed at the end of the cycle before the next round of DNA synthesis.

This model is obviously tentative and incomplete. It provides no explanation for the changes in u.v. resistance neither does it suggest how one of the two chromatids produced after DNA synthesis is initially inactive. It might be regarded as surprising that any distinction could be made between the two new copies of the genome after
DNA synthesis. But it is equally surprising to find that potential and rates of synthesis remain unchanged while a cell goes through DNA synthesis and division, and then change suddenly during interphase. It must also be remembered that yeast are eukaryotic cells with multiple chromosomes and probably nuclear histones (Tonino & Rozijn, 1966), and their chromosomal organization may be much more complicated than in prokaryotes.

It remains to be seen whether all the events that happen at the critical point are due to the same underlying cause or whether they are merely coincidental. It also remains to be seen whether this delay between chemical replication and functional replication is a peculiarity of yeasts, perhaps only of this yeast. But it does raise an important question for higher cells. An extra genome is made during the S-period: when is it first used? Does this happen immediately after it has been made (as in prokaryotes), or at some point in $G_2$ (as in $S. pombe$), or only when there are 2 new nuclei after mitosis?

The change of the sucrase $K_m$ on derepression suggests that 2 or more iso-enzymes are involved, and this could also be true of the other 2 enzymes. This does not affect the argument that has been presented: indeed it strengthens the concept of functional replication as a rapid process involving the whole genome. Tauro & Halvorson (1966) have suggested that the apparently continuous synthesis of an enzyme may be due to several small steps, which cannot be resolved, spread through the cycle and caused by the periodic transcription of separate structural genes for that enzyme. This interpretation is unlikely to be correct here since there would have to be many steps to account for the continuous increase through the cycle, and it also fails to explain the changes of rate and potential.

These changes in rate and potential seem to have an intimate connexion with gene replication, but this does not appear to be the case with the periodic enzyme synthesis which is the dominant pattern in both prokaryotes and eukaryotes. Periodic synthesis, for example, will continue after DNA synthesis has been blocked. The theories of 'oscillatory repression' and 'linear reading' which have been developed as explanations of this pattern of synthesis are discussed by one of us elsewhere (Mitchison, 1969a).

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Enzyme synthesis during the cell cycle


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STATISTICAL APPENDIX

We wish to determine which of two possible models best describes the changes in concentration of an enzyme during the growth of a given cell culture. Under the segmented model the enzyme concentrations will follow a curve consisting of three linear segments with the distance between the two rate-change points approximately equal to the generation time of the cells. Under the smooth model there will be no discontinuities in the rate of increase of enzyme concentration and under ideal conditions the concentration might be expected to increase exponentially.

The observations will not fit either curve exactly. Errors will arise in both the sampling procedure and the determination of the enzyme concentrations of the samples. Moreover the 'true' model can be at best only an approximation to reality. We therefore expect some variation between the observed concentrations and their values as predicted by the true model. We assume that these errors are independently and Normally distributed with constant standard deviation.

We therefore wish to discriminate between two regression models. If \( y \) is the observed concentration of enzyme at time \( t \) then the two alternative models are:

\[
\text{Segmented model } y = f(t) + \varepsilon, \tag{1}
\]

where

\[
f(t) = a + \beta_1 t \quad \text{for } t \leq T_1, \\
= a + \beta_1 T_1 + \beta_2 (t - T_1) \quad \text{for } T_1 < t \leq T_2, \\
= a + \beta_1 T_1 + \beta_2 (T_2 - T_1) + \beta_3 (t - T_2) \quad \text{for } T_2 < t,
\]

and \( \varepsilon \) is distributed Normally with standard deviation \( \sigma_\varepsilon \). The parameters \( a, \beta_1, \beta_2, \beta_3, T_1, T_2 \) and \( \sigma_\varepsilon \) are unknown. We impose the restraint that \( T_2 - T_1 > 90 \text{ min.} \)

\[
\text{Smooth model } y = g(t) + \varepsilon, \tag{2}
\]

where \( g(t) = a + b \exp (ct) \) and \( \varepsilon \) is distributed Normally with standard deviation \( \sigma_\varepsilon \). The parameters \( a, b, c \) and \( \sigma_\varepsilon \) are unknown.

The general problem of discriminating between two regression models has been discussed by Cox (1961). Our analysis is in principle identical to that suggested by Cox, but in place of his theoretical results, which are not applicable when one of the regressions is segmented, we have used results derived by simulation.

To illustrate our method of analysis we use as an example the 141 sucrase concentrations taken at 2.5-min intervals from a synchronous culture. A graph of the concentrations is given in Fig. 6 of the main text. Assuming in turn that each of the two models is true we first estimate the parameters by the principle of least squares.
Methods for fitting segmented regressions have been described by Hudson (1966). The exponential regression can be estimated by iterating on $c$ and fitting $a$ and $b$ by linear least squares.

The estimated line segments had slopes $0.48$, $0.89$ and $1.59$ with rate-change points at $118$ and $245$ min. The residual sum of squares (i.e. the sum of squares of differences between observed and fitted values) was $3023$, from which the standard deviation $\sigma_f$ was estimated as $4.73$.

The estimated exponential curve was $20.6 + 79.9 \exp (0.029t)$. The residual sum of squares was $3768$, giving an estimated standard deviation $\sigma_g$ of $5.23$.

It is seen that $\sigma_f$ is less than $\sigma_g$. The segmented model gives the better fit, but are we justified in concluding that the true model is segmented rather than smooth? To evaluate this we use the single statistic $\lambda$, the ratio of the two residual sums of squares. In this experiment $\lambda = 3023/3768 = 0.802$. Low values of $\lambda$ will arise if the true model is segmented, high values if the true model is smooth, but what do we mean by low and high? We need to determine whether the observed value of $\lambda$ is low enough to be compatible with the range of values expected if the segmented model were true, and also to confirm that such a value is unlikely to have arisen if the smooth model were true.

Let us first assume that the segmented model is true with parameters $a$, $\beta_1$, $\beta_2$, $\beta_3$, $T_1$ and $T_2$ given by our estimates. We can simulate a set of $141$ observations by calculating $f(t) + \epsilon$ for the $141$ values of $t$, where the $\epsilon$ are a set of $141$ independent random deviates from a Normal distribution with standard deviation $4.73$. The two regression models can be fitted to these simulated concentrations and the value of $\lambda$ determined. If this is repeated several times with different sets of random Normal deviates we can obtain an indication of the range of values of $\lambda$ compatible with the segmented model. Ten sets of simulations gave the following values of $\lambda$ arranged in ascending order:

- $0.684$
- $0.688$
- $0.695$
- $0.727$
- $0.754$
- $0.769$
- $0.775$
- $0.784$
- $0.820$
- $0.861$

The observed value of $\lambda$, $0.802$, lies within the range obtained when the segmented model is assumed true but well outside the range of $\lambda$ obtained when the smooth model is assumed true. We therefore conclude that the true model is segmented.

The above analysis was programmed for a computer and carried out for each experiment. The distributions of $\lambda$ are functions of the regression parameters and therefore have to be determined by simulation for each experiment independently.
Fewer than 10 simulations were sometimes sufficient to indicate which model was true but the results were not always as clear-cut as in the above example. In some cases the observed value of $\lambda$ did not lie within either range of 10 simulated values. In such cases the numerical value of

$$t = \frac{\text{observed log } \lambda - \text{mean of simulated log } \lambda s}{\text{S.D. of simulated log } \lambda s}$$

was calculated. If $t$ was less than 2 for one model but much greater than 2 for the other model then the former model was assumed true. This still left some cases in which the observed value of $\lambda$ was compatible with neither range of simulated values, so that neither model could be assumed true, and other cases in which the 2 simulated ranges overlapped and no discrimination between the two models was possible.

Graphical study of the increase in log concentration of alkaline phosphatase and acid phosphatase in some asynchronous control cultures suggested that the growth, though in general smooth, was not exponential. For these 2 enzymes therefore the smooth model was assumed to be a polynomial of degree 5, i.e.

$$y = a_0 + a_1 t + a_2 t^2 + a_3 t^3 + a_4 t^4 + a_5 t^5.$$ 

This gives a more general family of smooth curves which embraces approximately the exponential. The analysis otherwise was identical to that described for the sucrase example.

The values of $\lambda$ obtained by simulation depended on the assumption that the errors about the true regression model are Normally and independently distributed with constant variance. From a study of the residuals it was apparent that the variance was not constant, it increased with enzyme concentration, and that there was some serial correlation between the residuals. The effects of an increasing variance and of a serial correlation on the distribution of $\lambda$ was investigated by further simulation studies and found to be negligible for the purposes of the method of analysis used.

It is hoped to publish elsewhere a more detailed account of the statistical methods described in this appendix.

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