Aspects of Microbiology

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Preface

The purpose of this book is to show that the behaviour of populations of micro-organisms in batch cultures rarely reflects that of individual cells. I did not intend it to be a comprehensive summary of the state of current knowledge of the microbial cell cycle. Instead, I have attempted to provide the background to the main areas of the subject and, more importantly, to compare the cycles of prokaryotes and eukaryotes. There are omissions, but I have sought to cover the gaps by giving pertinent references, especially to review articles. The final chapter contains a personal selection of currently popular areas of study.

I am indebted to Dr J.A. Cole and Dr C.J. Knowles for their helpful comments during the early stages of this work, to Mrs J. Johnston and Mrs L. Marsh for preparation of the typescript, and to my wife Tisha for cheerfully helping with the correction of proofs.

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Contents

1 Introduction

The main aims of this book are to show that cellular events occur in sequences which can be revealed only by studying cells at known stages in the cell cycle; to review the methods which can be utilized to unravel cell cycle processes; to give examples of some of the major cellular processes which have been investigated—DNA and protein synthesis, for example; to demonstrate by means of commonly used systems how micro-organisms are being utilized to study various aspects of their cycle; and to attempt to point out some of the main differences and similarities which are apparent in the cell cycles of prokaryotic and eukaryotic micro-organisms.

A useful starting point is to look at the various terminologies commonly encountered in the literature. The *cell cycle* can be defined as a fixed period during which a cell, newly formed at division, grows until it divides to form two daughter cells. The duration of the cycle will depend upon the particular cell type and the prevailing environmental conditions. For example, a bacterium such as *Escherichia coli* can have a cycle time of 20 min in a complex rich medium whereas in simpler salts media containing a known energy source the cycle will be longer, assuming all other conditions such as temperature remain the same.

During each cycle, new cell material must be synthesized so that, at division, each daughter cell receives an equal portion of all components. It will be obvious that the cellular complement must at least double during the cycle otherwise it becomes diluted in successive generations. This synthesis of new materials must be integrated and regulated in an *ordered sequence* at the correct locations within the cell. Also, each cell component must be present at the appropriate time in the cell cycle. The cycle therefore has *spatial* and *temporal* controls which constitute a differentiation process at the cellular and molecular levels.

Traditionally, the various cellular processes of micro-organisms have been investigated in *batch culture*—that is, growth is exponential for a few generations until nutrients become depleted and then gradually ceases. *Continuous cultivation* of micro-organisms has also been employed, using *chemostats* (in which growth depends on the rate of supply of a limiting nutrient) or *turbidostats* (in which culture density governs the flow-rate of incoming nutrients). Both approaches attempt to standardize growth conditions and maintain the organisms at a fixed growth rate. The populations of both batch and

continuous cultures will consist of cells at all stages of the cell cycle ranging from young newly divided cells to older cells at the point of division. Such populations, therefore, can be said to consist of *asynchronously dividing cells*. Any event measured in these cells, such as bacterial wall synthesis, will reflect only a *time-averaged* value over the whole of the cell cycle. Newly divided bacteria may synthesize cell wall more rapidly than those at division but measurements of this parameter in asynchronous cells gives only a mean value and tells us nothing about patterns of synthesis during the cycle. To obtain further information about the sequence of events, the investigator must be able to obtain cells at a known stage of the cycle. Two approaches that can be used to achieve this involve studying single organisms or populations which are at the same stage of the cell cycle.

Measurements of cell cycle parameters in single cells are severely limited by the range of techniques that can be used. Biochemical determinations usually require much more experimental material. Parameters which can be assessed by microscopic examination include increases in volume and cell growth. This technique can be very accurate for those cells which grow only in length. A good example is the fission yeast *Schizosaccharomyces pombe*. It has been shown by microscopical measurements that an increase in cell volume of this yeast occurs only over the first three-quarters of the cell cycle.

Because of the obvious limitations of single-cell methods and the ever-increasing development of new biochemical techniques, a large number of methods have been devised for obtaining populations of cells which are of similar age and hence at the same stage in the cell cycle. One approach is to prepare *synchronous cultures*, in which cells are of similar age and which, therefore, grow and divide synchronously. Another approach is to separate cells from an asynchronous culture into age classes which, collectively, represent the whole cell cycle. This latter technique is called *culture fractionation*. These two approaches will be examined in greater detail in the next chapter. Suffice to say that none of the methods employed to obtain cells of similar age is free from criticism. More importantly, they are not universally applicable, since some of the methods have been developed for specific cell types: for example, light/dark cycles for inducing synchrony in photosynthetic organisms.

A whole range of cell cycle events can be measured once a system has been developed. Unfortunately, there appears to be a gulf between many areas of research and the use of cell cycle methods such as synchronous cultures. Perhaps the chief cellular component that has been studied during the cell cycle of both prokaryotes and eukaryotes is DNA synthesis. Other important processes, such as membrane biogenesis, cellular respiration and associated enzymes,

have been sadly neglected until recently. This is surprising in view of the known temporal stages undergone during DNA synthesis in both prokaryotes and eukaryotes. These stages are more apparent in eukaryotes since DNA synthesis is always periodic, occurring at a particular stage of the cell cycle known as the *S-phase* (synthetic phase). The integration of this event with other cellular processes has not been extensively studied.

Synthesis of various enzymes during the cell cycle of both prokaryotes and eukaryotes can occur continuously or periodically during the cycle. Patterns of synthesis vary for different enzymes or even for the same enzyme in different cell types. Measurements of enzyme levels during the cell cycle are fraught with difficulties. An assumption widely made in the literature is that enzyme activity is directly proportional to enzyme amount; unfortunately this may be an oversimplification. Another factor which influences interpretation of enzyme activity during the cell cycle is that of *enzyme potential.* Observed enzyme activity need not reflect the maximum possible activity at a given stage in the cycle. This problem is best illustrated by examples of inducible enzymes such as ß-galactosidase. In the repressed state very little enzyme activity will be present but this may increase a thousandfold in the fully induced state. Other parameters affecting enzyme activity *in vitro* include the presence of intracellular proteases and loss or dilution of an essential co-factor during assay. Unfortunately these considerations are not always taken into account in studies of patterns of enzyme synthesis during the cell cycle.

Both prokaryotic and eukaryotic micro-organisms can usually be cultured conveniently and rapidly in large amounts in easily prepared growth media. They provide a whole spectrum of cellular processes, each of which can differ between species. This diversity and ease of cultivation makes micro-organisms particularly suitable for investigating cell cycle events.

The choice of a particular organism will depend on a variety of criteria—the ease of establishing it in synchronous culture for example—but it is usually influenced by a particularly interesting property of the physiology of the organism. Rod-shaped bacteria such as *Bacillus subtilis* have been used extensively to study surface growth characteristics during the cell cycle. Other morphologically differentiating bacteria such as *Caulobacter crescentus* are suitable for investigating flagellar biogenesis and synthesis of phage attachment sites during the cycle. In suitable culture media, pigmented prokaryotes such as *Rhodopseudomonas palustris* can be utilized to further our understanding of bacterial photophosphorylation when they are grown in the light, and of oxidative phosphorylation when they are grown in the dark. This particular prokaryote has the added bonus of a dimorphic cell cycle giving rise to a swarmer cell and a sedentary

tubed cell. Other bacteria are useful in cell cycle studies because of their extreme adaptability and ease of genetic manipulation. A noteworthy example of such a genetically well-defined prokaryote is *Escherichia coli.*

Eukaryotic micro-organisms also provide a variety of cell types which have been utilized in studies of the cell cycle. Yeasts have been used extensively in this respect, especially *S. pombe*, which is readily established in synchronous culture by a variety of methods. Another commonly used yeast is *Saccharomyces cerevisiae.* The yeasts make useful systems for investigating respiratory development during the cycle because they can be grown fermentatively or non-fermentatively. Protozoa such as *Tetrahymena pyriformis* and the amoebae, which tend to be susceptible to lysis after relatively mild disruption procedures, make suitable cell systems for following the development of intra-cellular organelles during the cycle. Unicellular algae such as *Chlorella* can be utilized to investigate photosynthetic reactions.

These are potentially exciting experimental systems for increasing our knowledge of cellular physiology. However, enthusiasm should be tempered by an awareness of the danger of applying an understanding of a process in one organism to that in another. Micro-organisms are notoriously wayward entities which can carry out what is superficially the same process *via* different pathways or by utilizing different enzymes. This complexity may be manifest in a single organism and not confined to inter-species differences. A good illustration of this is electron transport in aerobic bacteria: a single bacterium such as *E. coli* can oxidize reduced substrates using different pathways of electron flow and respiratory carriers, depending on the prevailing growth conditions.

2 Methods for investigating the cell cycle

Every stage of the cell cycle will be represented in a growing asynchronous culture. Yet some events (such as DNA synthesis) are confined to discrete periods of the cycle. Hence, in order to study such events it becomes necessary to work with cells of similar age and follow them through the phases of their cycles. Unfortunately, this is not achieved simply. We shall see that the methods used to prepare cultures of micro-organisms homogeneous with respect to age are rarely free from criticism. The main objection is that they perturb the treated cells, giving rise to artefacts and abnormal cell cycle events. Also, certain species of micro-organisms are suitable only for specific methods for obtaining cells of different ages. Perhaps the best example is the regime of alternating light and dark periods which induces synchronous division of photosynthetic micro-organisms. In this chapter we will concentrate on methods of preparing synchronous cultures, the separation of asynchronous cells into age classes by culture fractionation, and naturally occuring synchrony.

Preparation of synchronous cultures

Over the past few years many techniques have been introduced for preparing cultures of synchronously dividing micro-organisms. Some work well only for one type of organism whereas others have wider applications. The purpose of these methods is to yield a population of cells which is relatively homogeneous with respect to age and which grows and divides synchronously.

Assessment of the extent of synchrony A synchronous culture is one in which all the cells are at the same stage of the cell cycle, so that they grow and divide together. Such a culture amplifies the phases undergone by a single cell. Theoretically, all the cells of a synchronous culture should grow and divide at the same time, giving rise to a stepwise increase in cell numbers. In practice, this is never achieved: the division period is prolonged because of slight inherent age differences between cells (Fig. 2.1). The shorter the division period, the higher the degree of synchrony. Various indices have been developed to assess how close particular synchronous cultures come to the ideal or theoretical case. Such indices also allow us to compare the degree of synchrony in different experiments and to determine the efficiency of the various methods used to achieve synchronous

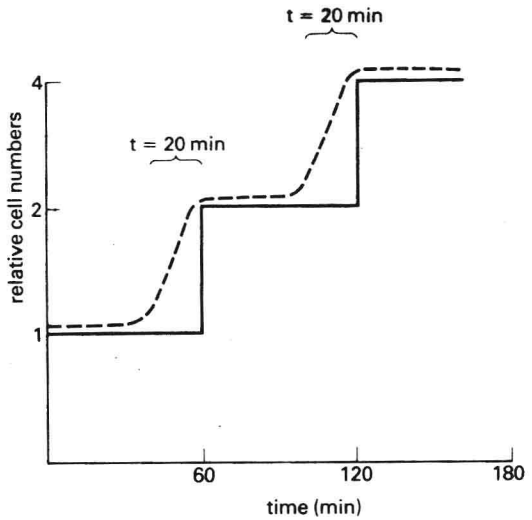

Fig. 2.1 Comparison of idealized synchronous growth (————) with that obtained in practice (– – – –).

growth. It is important to use the same index for all experiments because application of different indices can give quite different results.

An often used index is that of Blumenthal and Zahler (1962), which is given by

$$F = N/N_0 - 2^t/g$$

where F is the synchrony index, N_0 and N are the numbers of cells before and after division respectively, t is the time in minutes taken for division, and g is the *generation time* (the time in minutes for all the components of the culture to double in amount). This index takes into account both the extent to which doubling has been achieved (ideally N/N_0 should equal 2.0) and the time taken for a synchronous burst of division compared with the generation time of cells during exponential growth.

For a perfect synchronous culture F will be 1.0. For the experimental culture in Fig. 2.1, the generation time is 60 min, the time taken for division is 20 min, and $N/N_0 = 2.0$. This gives an F value of 0.75. An asynchronous culture with a doubling time of 60 min would have an F value of 0.0 as assessed by the above index. In practice, an index between 0.5 and 0.7 indicates an acceptably high degree of synchrony.

Balanced growth A central dogma of investigations using synchronous cultures is that the organisms should exhibit *'balanced growth'*. This requires that all measurable parameters, such as cell numbers, dry weight and respiratory activity, should increase by the same amount over the same time interval. Thus, in every cell cycle, a doubling of all the components should be observed. Because of discontinuities of synthesis of some cell constituents during the cell cycle (DNA replication, for example) the time taken for one cycle to be completed is the minimum interval that can be used to assess the extent of balanced growth.

Techniques developed for preparing synchronous cultures can be grouped into either *selection methods* or *induction methods*. These have been reviewed by Mitchison (1971) and Helmstetter (1969), respectively. Normally, as we shall see later, selection methods give rise to fewer metabolic disturbances and hence more 'balanced growth' than do induction methods. However, much information may be gleaned by deliberately perturbing the normal cell cycle using an induction method or by drastically modifying the growth conditions. Furthermore, adequate control experiments should always be carried out under conditions as near as possible to those used to prepare a synchronous culture. These should include measurements of the desired parameter in a culture in the exponential phase of growth as well as in a culture treated in a similar fashion to that used to obtain a synchronous culture. For example, cells separated according to size on sucrose gradients should be re-mixed to form the original asynchronous population. Unfortunately some methods, such as those which call for the use of inhibitors, are not amenable to adequate control experiments.

Presentation of data Because of the slight variation in division times of synchronously growing cells, it is important to be able to delineate the cell cycle in a reproducible fashion. Usually, the duration of the cycle is estimated from the mid-point of the first division to the mid-point of the second, and represented as a linear scale 0–1.0. Measurements of timing of events during the cell cycle are related to these points and adjusted to fall on the linear scale. For example, in Fig. 2.2a, oxygen uptake rates are shown to oscillate, rising to two maxima per cell cycle. From the mid-point of the first division to the mid-point of the second gives a cycle time of 75 min. We assign the first mid-point as 0 on our linear scale and the second as 1.0. Thus, each 0.1 unit of the linear scale is equal to 7.5 min. The first peak in the oxygen uptake rate occurs 25 min after the first mid-point and, therefore, at 0.33 of a cycle. The second peak in the oxygen uptake rate occurs 60 min after the first mid-point and, therefore, at 0.80 of a cycle. These results can be summarized as a cell cycle map, which is

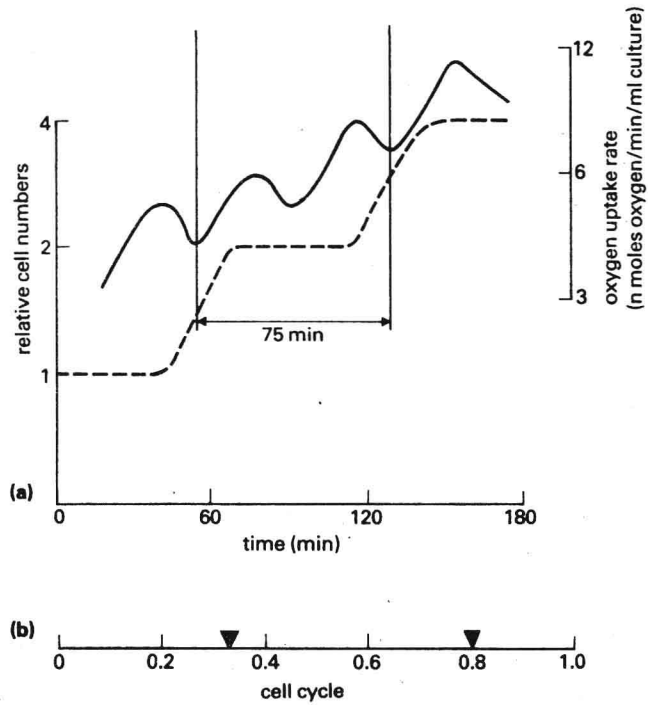

Fig. 2.2
a Theoretical pattern of oxygen uptake rates (————) during synchronous growth of a micro-organism (– – – –).
b Cell cycle map summarizing the timings of the maxima of oxygen uptake rates during the cycle. The cell cycle is represented as a linear scale from 0 to 1.0 which corresponds to the time from the first to the second mid-point of increase in cell numbers.

also shown in Fig. 2.2. By repeating the experiment a number of times, all timings of maxima of oxygen uptake rates can be represented on the map and the mean calculated. This method of presentation compensates for small variations in cycle times between successive experiments, and cycle maps provide a standard method of representing periodic events.

Induction methods

Induction methods involve the treatment of asynchronously dividing cells over a period of time in order to bring them into the same phase of the cell cycle. Some induction methods rely on deliberately

perturbing or shocking the culture and throw serious doubt on the normality of the resulting synchronous division and cell cycle events.

Induction based on end-points of growth Micro-organisms continue to grow only as long as their nutritional requirements are met. Once an essential growth factor is depleted, growth stops. This can be observed during the transition from exponential growth to the stationary phase of a batch culture. Often more than one growth factor will have been depleted and it is difficult to identify which is the first factor to become limiting. However, for some bacteria, resuspension of cells from the end of exponential growth into fresh growth medium causes a synchronous pattern of division for two or three cell cycles.

This method does not have universal application: it is successful for only a few species such as *B. subtilis*, *E. coli*, *Proteus vulgaris*, and some yeasts. The precise point at which organisms can be removed and resuspended in fresh growth medium to initiate synchronous growth is important. As can be seen from Fig. 2.3, cells in the late

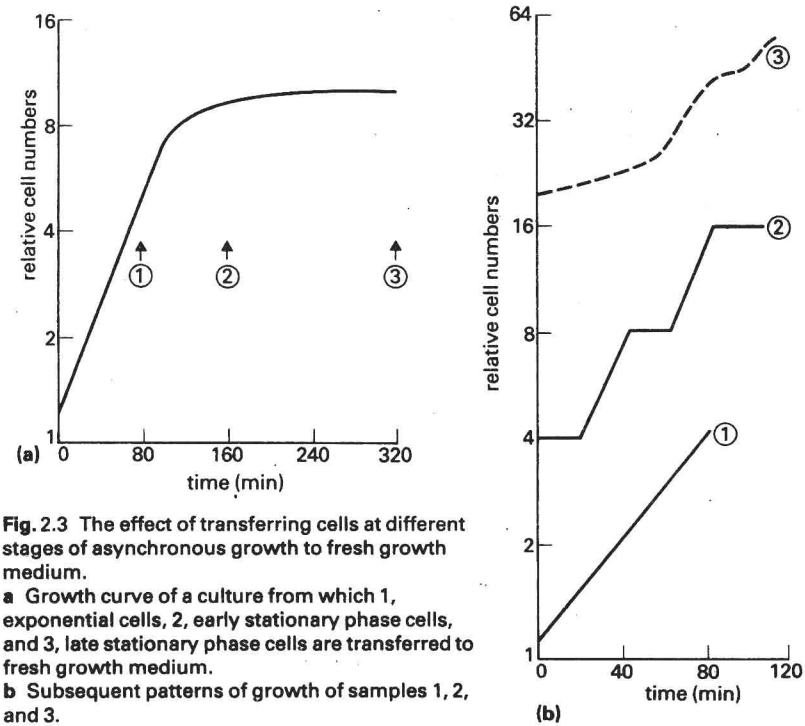

Fig. 2.3 The effect of transferring cells at different stages of asynchronous growth to fresh growth medium.
a Growth curve of a culture from which 1, exponential cells, 2, early stationary phase cells, and 3, late stationary phase cells are transferred to fresh growth medium.
b Subsequent patterns of growth of samples 1, 2, and 3.

exponential phase of growth usually continue to divide exponentially when resuspended in fresh nutrients, whereas cells at the onset of stationary phase (culture 2 in Fig. 2.3) divide synchronously. Major criticisms of this method are that the resuspended cells exhibit an abnormally long cell cycle which is poorly defined—that is to say, the exact mechanism by which cells are arrested by nutrient depletion is not known.

Another starvation method employed for obtaining synchronous division of *Bacillus* species is based on the synchronous germination of spores. This method probably works because the spores represent a homogeneous population which are similar in composition and hence will germinate synchronously. Little work has been done to demonstrate that events occurring during synchronous spore germination reflect those which occur during the cycle of vegetative cells.

The resuspension of stationary-phase cells into fresh growth medium and the synchronous germination of spores are examples of poorly defined methods for studying the cell cycle because neither the cause of synchrony nor the effect on the subsequent cell cycle is known. Other starvation methods which have more defined effects rely on induction of cell synchrony by the removal of *one* known essential growth factor from an exponential culture. However, the method succeeds only if the growth of each individual cell stops at the same stage of the cell cycle. Perhaps the commonest example of this approach is amino acid deprivation of amino acid auxotrophs. The essential amino acid is withdrawn for approximately one generation time and then added back to the culture. After re-supplementation, synchronous growth will occur only if *each cell was arrested at the same point in the cell cycle during the withdrawal period.*

Vitamin deprivation, such as the deprivation of thiamine from cultures of the flagellate protozoon *Polytomella agilis*, has been used to prepare synchronous cultures. An unusual specific starvation regime is used to prepare synchronous cultures of diatoms. These organisms require silicon to form the shell which encloses the cells. Silicon deprivation for a fixed period from a culture of the diatom *Cylindrotheca fusiformis*, followed by its restoration, has been employed successfully to prepare synchronous cultures of this organism.

Induction by hypoxia The deprivation of oxygen (*hypoxia*) from an asynchronous culture of an aerobic organism, followed by its reintroduction, can induce some micro-organisms to divide synchronously. This approach has been used to induce synchrony in cultures of the protozoon *Tetrahymena pyriformis*. The technique involved the application of hypoxia for a period of 4 h, causing cells to accumulate in the G2 phase of the cell cycle (see page 40, Chapter 3). When

aeration was resumed, synchronous division occurred. However, it must be appreciated that such a method can induce profound physiological changes, especially with strictly aerobic micro-organisms. For example, it is known that under such conditions micro-organisms undergo changes in their respiratory chains and hence their capacity for energy production is also modified.

Induction by inhibitors Various chemicals can block asynchronously growing cultures at definite stages of the cell cycle. If they are exposed to the inhibitor for at least a generation time, then theoretically *all* the cells of that culture will progressively become blocked at the same stage of the cell cycle. If the inhibitor is then replaced by fresh growth medium, synchronous growth will commence. A good inhibitor for induction of synchronous cultures must be specific for a definite phase of the cell cycle; must block *all cells immediately* they reach this stage of the cycle; must be removed readily by washing procedures; and must not damage or kill a proportion of the culture.

Commonly used inhibitors are those which block DNA synthesis. These are especially suitable for eukaryotic micro-organisms in which DNA synthesis is confined to the S-phase (see page 40, Chapter 3). One such inhibitor, hydroxyurea, has found wide application in inducing synchronous growth in mammalian cells, and in micro-organisms such as the trypanosomatid *Crithidia luciliae*, and in the yeast *S. pombe*. Unfortunately, this inhibitor may be selectively lethal during the S-phase of some organisms so that, on its removal, the culture contains a proportion of non-dividing cells.

Deoxyadenosine is of interest because it inhibits DNA synthesis without greatly affecting RNA synthesis or increases in dry weight. It has found wide application in mammalian cells and micro-organisms. In the fission yeast *S. pombe*, it has been used by treating a growing asynchronous culture with 2mM deoxyadenosine for 3 h, slightly longer than the usual 2.5 h doubling time of this organism (Mitchison and Creanor, 1971). On removal of the inhibitor, there is a burst of DNA synthesis followed by a synchronous division. Dry weight and RNA synthesis continue during treatment causing greater variation in size and mean size of cells before the first division than is the case in asynchronous cultures.

These observations have led to the concept that there are two cycles—the *DNA division cycle* and the *growth cycle*. Normally both are linked, but when DNA synthesis becomes blocked with deoxyadenosine, the DNA-division cycle is inhibited while the growth cycle (RNA, dry weight) continues. This dislocation of the two cycles leads to an abnormal size distribution before the synchronous division that occurs after removal of deoxyadenosine. On addition of the inhibitor,

those cells in S-phase will be inhibited immediately, whereas those which have just completed S-phase will continue to grow and divide until they again reach S-phase. The latter class of cells will be approximately the usual size for this stage, but the former will continue to grow during the arrest period and become abnormally large. Therefore, treatment with deoxyadenosine will synchronize the DNA-division cycle but *not* the growth cycle. This ability to dissociate the two cycles provides a powerful manipulative tool for comparing events during 'normal' cell cycles in selection-synchronized cells in which both the DNA-division cycle and growth cycle are synchronous with those in the deoxyadenosine-induced synchronous DNA-division cycle.

Induction by periodic supply of growth factors This approach is a refinement of the technique for inducing synchrony by starvation. Methods have been developed, largely with chemostat cultures, for periodically pulsing an essential growth factor to an asynchronous culture. The important requirements are that the time between each pulse ideally should be the normal generation time for the given growth medium, and that the growth factor pulsed is usually enough to maintain growth for one doubling. Most methods rely on periodic pulsing of the carbon source as the limiting growth nutrient but other growth factors such as various amino acids have also been used.

The method has been used to induce synchronous growth in a strain of *E. coli* (Buckley and Anagnostopoulos, 1975). Briefly, the method involves growing the bacteria in salts medium containing a limiting amount (0.25 g per litre) of glucose. Starting with an asynchronous culture of 100 ml, 50 ml of fresh growth medium are added every cycle time, which may be more, less, or the same as the generation time of cells during growth in batch culture. On addition of fresh medium, 50 ml of the total volume (150 ml) are removed, thus maintaining a constant volume of 100 ml. Continuous cycling in this manner eventually leads to synchronous division of the cells, ascribed to a blockage of DNA replication during the pulsing treatment. Further work is required to check the validity of this method, since it is possible that only the DNA-division cycle is synchronous, while the growth cycle is not. It is not known whether the events occurring during periodic pulsing are the same as those occurring in cell cycles of synchronous cultures established by other methods, especially selection methods. The technique has the attractive feature of altering the duration of the cell cycle by varying the time between pulses, and also of establishing a continuous synchronous culture system.