NUCLEIC ACID SYNTHESIS IN THE GROWTH CYCLE OF THE FISSION YEAST, SCHIZOSACCHAROMYCES POMBE, AND OTHER ORGANISMS

by

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Chapter 1

General Introduction

It is now generally accepted that the genetic information of cells is encoded in the sequence of bases in the Deoxyribonucleic Acid (DNA) contained in the nucleus and other organelles. This knowledge allows us to ask specific and more informed questions about the control of synthetic events that occur in biological systems. There are many levels at which the answers can be sought, but in this discussion we shall confine ourselves mainly to studies designed to investigate the temporal relations between discrete events, with the possible inference of causal relationships, which occur during the cell cycle.

Cells undergo cyclic changes, there being periods of growth when the various macromolecular components are doubled in quantity, and periods of division. Initially work on this cycle centred on the division process, which is the most dramatic and easily observed event within this cycle, without much attention being paid to events occurring between divisions. The problem confronting people was one of identification of patterns of synthesis or discontinuous events occurring between divisions of randomly growing cells. The microspectrophotometric and autoradiographic techniques on single cells to some extent overcame this problem, but they have the severe

limitation that, on the whole, only gross macromolecular syntheses can be observed. The synchronous culture is a major step forward from the single cell analyses, because it permits the biochemical investigations of individual molecules, but it has the disadvantage of being difficult to relate to the randomly growing culture from which it has been derived.

The type of organism which is suitable for cell cycle analysis is that which is amenable to both these lines of investigation. There are many organisms which fit these qualifications, though, due to various disadvantages, none of them is ideal. Here in Edinburgh, a lot of work has been carried out into various aspects of the cell cycle of Schizosaccharomyces pombe, using both the single cell and the synchronous culture techniques. S. pombe is one of three species of fission yeast of the genus Schizosaccharomyces, which, together with the closely related genus Saccharomyces, belongs to the class Ascomycetae, one of four classes of the true fungi (Smith, 1955).

What do we mean by 'the cell cycle'? The concept has evolved from the mitotic cycle, which was a term used to describe the phenomenon of growth and division in cells, but which, as the name implies, placed greater emphasis on division (Hughes, 1952). A cycle is a period of time in which events happen in a certain order, and which is constantly repeating itself. Division as such is therefore only

represented once in each cycle, as are all the other biochemical events necessary for growth, and it should not, therefore, be given any undue emphasis. Division does serve a very useful function, though, as a reference point to which the other events may be related, because of the ease with which it can be observed. In S. pombe there is the problem of which point in the cycle one considers the cell to have divided. It could be either the physiological separation of the cell by the formation of the cell plate (Swann, 1962), or the physical separation of the two daughter cells by fission (Mitchison, 1957). It is of little consequence which of these two is taken as the reference point so long as it is clearly specified, otherwise confusion can arise when the relationship between different cell cycle phenomena is considered. Throughout this thesis fission will be taken as the reference point, such that it is represented by either 0.0 or 1.0 on a cell cycle map extending from 0.0 to 1.0. The first appearance of the cell plate is therefore at 0.85 and nuclear fission at about 0.75 of the way through this cycle (see figure 1).

The first cell cycle studies on S. pombe were concerned with the changes in dry mass, volume and concentration (Mitchison, 1957). Mitchison showed that the rate of increase in dry mass is constant throughout the cell cycle, but that this rate doubles sharply just before fission. The rate of increase of volume, on the other hand, increases throughout the cycle until

nuclear division occurs, when the rate falls to zero and the cells enter a constant volume stage. During the constant volume stage the cell is synthesising the cell plate, so the former is probably a consequence of the latter.

Dry mass and volume are very broad parameters, the former, for example, describing the total molecular composition of the cell. Dry mass was then examined in a little more detail. The pattern of incorporation of labelled precursors into protein, ribonucleic acid (RNA), and carbohydrate was examined (Mitchison and Lark, 1962; Mitchison and Wilbur, 1962). It was shown that the synthesis of these macromolecular components is exponential throughout most of the cycle. Exponential synthesis of protein and RNA have since been confirmed by Stebbing (pers. commun.) using bulk analyses on synchronous cultures. The dilemma between the exponential synthesis of the main macromolecular components of the cell and the linear synthesis of total dry mass was accounted for by the postulated fluctuating acid soluble pool (Mitchison and Wilbur, 1962). That this is indeed the case was shown later by Mitchison and Cummins (1964).

Total RNA is not only divisible into messenger (m-RNA), transfer (t-RNA) and ribosomal (r-RNA) RNAs, but also into the many different types of molecules within each of these classes. Total protein can also be subdivided into many different fractions. The measurement of total RNA and protein will

therefore only yield the composite pattern of synthesis of these many subdivisions.

There is, however, more detailed information about the synthesis of these two types of macromolecules. Pulse labelling of randomly growing cultures with 3H-adenine, for different lengths of time, indicated that the pattern of synthesis of the different types of RNA were essentially similar (Mitchison and Lark, 1962). The short pulses of 3H-adenine enter a rapidly labelled nuclear RNA, whereas the longer pulses tend to label primarily ribosomal RNA in the cytoplasm. The relationship between the rapidly labelled nuclear RNA and messenger RNA is not clear, because a large proportion of the former never enters the cytoplasm, its synthesis and breakdown being limited to the nucleus (Harris, 1968). Perhaps it is all messenger RNA, only a small fraction of which gets into the cytoplasm, the factor controlling the rate of entry into the cytoplasm being another level of control in the expression of genes (Harris, 1968). Whether the rate of synthesis of messenger RNA is related to the rate of synthesis of the rapidly labelled nuclear RNA will depend on whether there is any 'selection' of individual messenger RNA molecules before they are able to enter the cytoplasm. In the stepdown situation, because there is little or no net synthesis of r-RNA, and the nuclear RNA, which is similar in base composition to the DNA, subsequently appears in the cytoplasm, there is

greater evidence for believing that the nuclear label in the short pulses might represent the synthesis of messenger RNA (Mitchison and Gross, 1965; Mitchison 1963b). In these studies the incorporation of labelled bases into RNA during stepdown suggested that messenger RNA is synthesised at a constant rate throughout the cell cycle. A necessary consequence of this result is that the rate of synthesis must double at the time of division. Essentially this is as far as the information about RNA synthesis goes, except for some results of Vincent (1965) concerning the synthesis of r-RNA and its assembly into ribosomes. These suggest that the rate of synthesis of r-RNA is cyclical, reaching a maximum between 0.5 and 0.75 of the cycle and a minimum at 0.3 of the cycle. Further, that there is a burst of ribosomal assembly also between 0.5 and 0.75 of the cell cycle. Total RNA and the 10 - 20 minute labelling pulses are, in essence, measuring r-RNA because of its vast excess, so it is difficult to reconcile these apparently different patterns of synthesis (Mitchison and Lark, 1962; Stebbing, pers. commun.).

The development of a technique for obtaining synchronous cultures of S. pombe has enabled studies of the patterns of synthesis of different proteins, which is not possible to do by the analysis of single cells. The synchronous culture of S. pombe has, in a short time, yielded some interesting results. Basically there are two patterns of protein synthesis. The

first is the 'step' synthesis shown by aspartate transcarbamylase (ATCase) and ornithine transcarbamylase (OTCase), where the synthesis is limited to short discrete intervals in the middle of the cell cycle (Bostock et al., 1966) and by tryptophan synthetase (TSase), the synthesis of which is restricted to the end of the cell cycle (Robinson, 1966). The other pattern is shown by repressed sucrase or acid phosphatase and the constitutive alkaline phosphatase, which are synthesised linearly through the cell cycle, but with sharp doublings in the rate of synthesis in mid cycle. It is also found that, if samples are taken from cultures in which sucrase is repressed and treated in such a way as to derepress the enzyme, the rate of synthesis of the derepressed sucrase also doubles in mid cycle (Mitchison, 1967).

This is only a brief summary of the biochemical events about which there is information in S. pombe. So far the discussion has revolved around RNA, protein, carbohydrate, volume and dry mass, and no mention has been made of the behaviour of DNA. At the time that the research for this thesis was started nothing was known about the DNA synthesis cycle in this organism. But DNA must be fundamentally important in the control of the synthesis of the macromolecules described so far, and thus, if we are to understand the control mechanisms involved, it is essential that we have knowledge of the DNA synthesis cycle. There are many questions that arise

from the work that has been described concerning the relationships between the synthesis of DNA and the other macromolecular cellular components.

Let us first consider the patterns of RNA synthesis. The linear increase in the rate of RNA synthesis over most of the cell cycle might suggest that there is a continual synthesis of DNA throughout the cell cycle, creating a constant increase in the number of templates available for RNA transcription. A close relationship between the rate of RNA synthesis and the amount of DNA has been shown in mouse fibroblasts (Killander and Zetterberg, 1965), Paramecium (Kimball and Purdue, 1962) and Tetrahymena (Prescott, 1960). In Saccharomyces cerevisiae the rate of RNA synthesis increases during the late S and early G2 phases (Williamson, 1965) and is related to the amount of DNA present in the culture. Measurements of total RNA in synchronous cultures of this yeast show that the net synthesis of RNA begins at the time of DNA synthesis and continues to about the time of cleavage (Williamson and Scopes, 1960), after which time there is no further increase in RNA until the following S period. In other organisms there is no relationship between the DNA content and the rate of RNA synthesis. In Physarum polycephalum, for example, the rate of RNA synthesis shows two peaks during the G2 phase (Mittermayer et al., 1964) and HeLa cells show an increase in the rate of RNA synthesis over the majority of the cell cycle, while the synthesis of DNA

is restricted to a part of interphase (Scharff and Robbins, 1965).

There is a slight indication that the rate of RNA synthesis might be constant in the last part of the cell cycle of S. pombe; the curves of ^{3}H-adenine incorporation flatten out over this period (Mitchison and Lark, 1962). If this is a real phenomenon it could be due to one of three factors. Firstly there is the evidence of Stebbing (pers. commun.) from bulk measurements of RNA in synchronous cultures grown on Edinburgh Minimal Medium 1 (see appendix 1). He shows that there is an exponential synthesis of RNA throughout most of the cycle, but that the rate of increase of RNA decreases at about the time of division, and is maintained at this lower rate during the next cycle. Such an effect would show up on autoradiographs as a tendency for the rate of incorporation of precursors to plateau at the end of the cell cycle. Secondly if the DNA is being synthesised for the majority of the cycle then this plateau might represent the point at which all the DNA templates had completed replication and RNA synthesis was proceeding at a maximum rate. On the other hand there is evidence that the rate of RNA synthesis is depressed at the time of DNA replication (Moses and Taylor, 1955; Nygaard et al., 1960; Sisken, 1959; Taylor, 1960; Prescott and Kimball, 1961), and the plateau may reflect a relatively short period of DNA synthesis towards the end of the cycle. The experiments on Euplotes (Prescott and Kimball,

1961) elegantly show that the process of DNA synthesis excludes the synthesis of RNA on that part of the chromosome that is replicating. However, equally well it shows that RNA synthesis continues in all other parts of the Euplotes macronucleus, so the net effect is small over the whole nucleus.

The step-down experiments also raise some interesting points. If the rate of RNA production is to some extent a function of the templates available for transcription, then the rapid doubling in the rate of synthesis at the end of the cycle might represent a doubling in the number of sites available for transcription. This is probably a more valid hypothesis for the step-down situation than in normal growth because the cell is transferred from a complex medium, rich in the precursors for macromolecular synthesis, to one in which it is required to produce them itself. This process might result in the need for new proteins and as such the synthetic machinery might be expected to be functioning at a maximal rate. In this condition the number of transcribing sites on the DNA might well be limiting. It should be borne in mind that the limiting factor need not necessarily be the absolute quantity of DNA present in the cell, it could be the availability of the DNA for transcription. Further experiments with tritium labelled bases do not all show the same pattern of incorporation (Mitchison, 1963b). Apart from the explanations discussed by Mitchison another possible explanation might be that the fall in

the rate of incorporation of ^{3}H-uracil reflects a fall in the rate of mRNA synthesis due to a concomitant synthesis of DNA. Though there is no evidence to show that DNA synthesis continues after step-down in S. pombe, Kjeldgaard et al. (1958) show that it continues for a short time in Salmonella typhimurium. The simultaneous increase in the rate of incorporation of ^{3}H-adenine might then be due to the incorporation of this precursor into DNA. After step-down the rate of incorporation of RNA precursors is only 5% of that in normally growing cells (Mitchison, 1963b). Therefore the ratio of RNA being synthesised to DNA is reduced to about 5:1. If, however, DNA synthesis is restricted to a limited part of the cycle the amount of DNA being synthesised at any instance during this period could well be in excess of the RNA being synthesised during the same time, and as such would have an effect on the pattern of incorporation of labelled precursors.

The sudden doubling in the rate of increase in dry mass is at first sight an interesting feature, because it is so clearly defined. But this observation loses some of its significance to this discussion when one considers that it is a function of the exponential increase of the major macromolecular components of the cell and a fluctuating pool. An exponential synthesis can give little guidance as to the possible pattern of DNA synthesis, except that which has already been mentioned. It is unlikely that the fluctuations in the total acid soluble

pool will show any close correlation to the timing of DNA synthesis.

The synthesis of individual proteins is likely to yield more useful information. With the exception of tryptophan synthetase, which steps at around the time of cell plate formation, the points (critical points) at which the amount, or rate of synthesis, of the enzymes investigated so far double, occur at roughly the same point in mid cycle. It is tempting to suggest that these doubling points represent the replication of the DNA which is coding for these enzymes (e.g. Gorman et al., 1964; Donachie, 1965). In bacteria there is fairly convincing evidence that the doubling in the rate of inducibility of an enzyme is correlated with the sequential replication of the genome. Though such a relationship may exist in bacteria for this type of protein synthesis, the same cannot be said for the 'step' enzymes, which can occur in the absence of any DNA synthesis (Masters and Donachie, 1966; Steinberg et al., 1965; Steinberg and Halvorson, 1968; Ferretti and Gray, 1967). It would be of interest to see the relationship between the biochemical replication of the encoded information in DNA for a gene product and the phenotypic replication of the gene in S. pombe.

These are some of the points which, in order to be clarified, require a knowledge of the pattern of DNA synthesis. There is another aspect of the cell cycle work on S. pombe

which is closer to the problem of DNA synthesis. This is the work of Swann (1962 and pers. commun.) and Gill (1965) who have looked at the effect of ultraviolet irradiation and ^{32}P suicide decay during the cell cycle.

The sensitivity of S. pombe to ultraviolet irradiation, measured by the dose required to kill 50% of the cells, shows dramatic changes over the cell cycle. From 0.9 to 0.2 in the next cycle cells are sensitive, but this period is followed by a rapid rise in resistance, which reaches a maximum at 0.4. This resistance is maintained until 0.6, after which it steadily declines to the beginning of the sensitive phase (Swann, 1962). In a similar analysis of the effect of ^{32}P suicide decay, Swann (pers. commun.) has shown that the periods of sensitivity and resistance occur in almost identical phases of the cycle. In an analysis of sensitivity to U.V., as measured by division delay, Gill (1965) showed that there are two distinct phases. There is a major period of sensitivity between 0.5 and 0.1, and a second period of minor sensitivity between 0.1 and 0.5. Though there is a discrepancy in the precise timing of these periods of sensitivity, the shapes of the curves are essentially the same and Gill argues that the difference in timing is merely a consequence of the different methods of assessing the stage of the cycle in which a cell is.

Swann has gone on to look at the efficiency of different wavelengths of ultraviolet light in killing cells at different

stages in the cycle. He shows that deaths in 'sensitive' cells demonstrate an action spectrum characteristic of nucleic acids, whereas those in the 'resistant' phase show one characteristic of protein (Swann, pers. commun.).

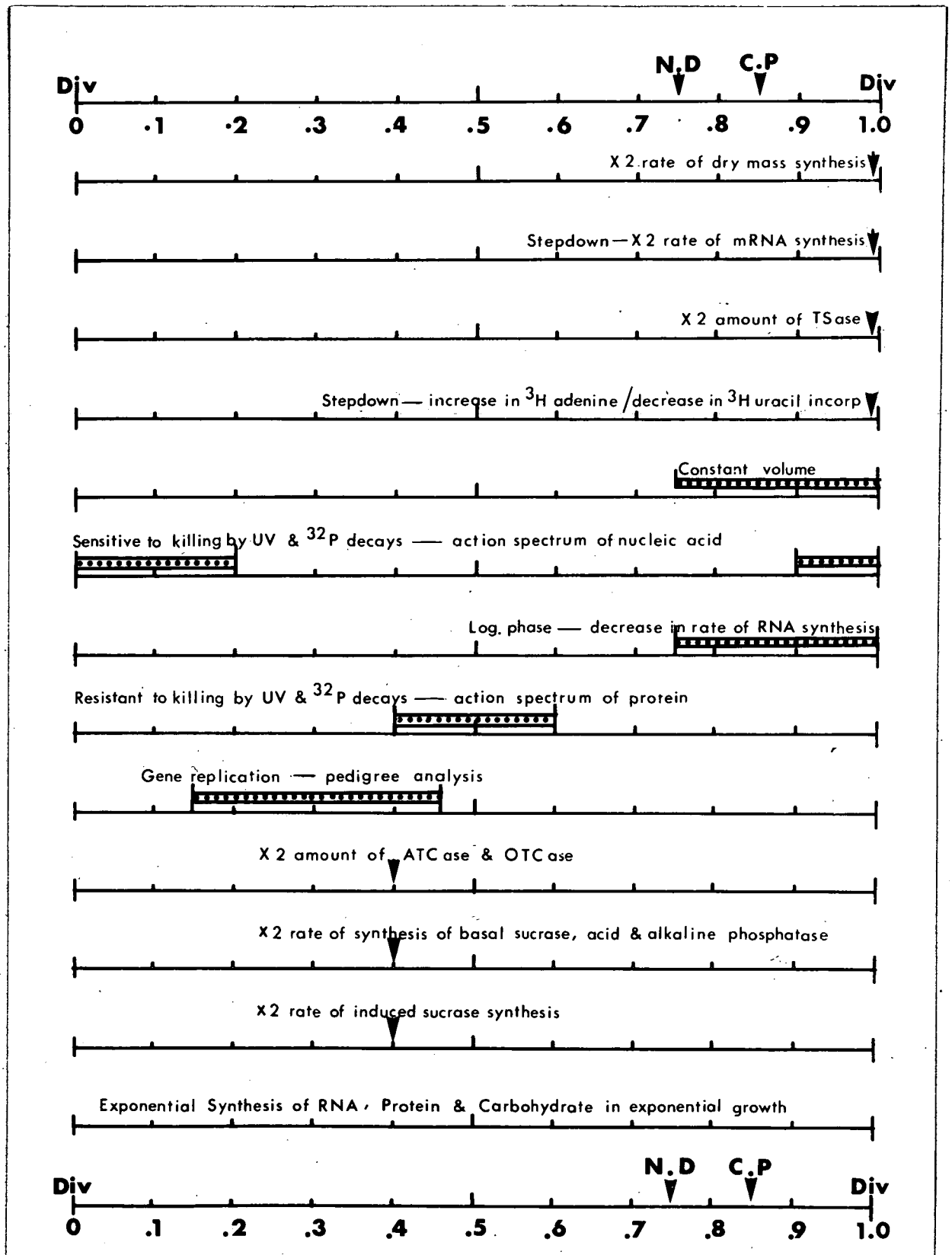
Added to these phenomena are the results of the pedigree analysis following irradiation (Swann, 1962). Here the change over from similar to differential deaths in daughter cells occurred between 0.15 and 0.45 in the parental cycle. The conclusion drawn by Swann from these results was that gene replication occurs at this point. These results will be discussed again in the final chapter, but it is helpful to ask a few questions at this stage. Is the period of 'gene' doubling, which Swann suggests takes place at 0.4 of the cycle, synonomous with DNA replication? If it is, it is interesting that the action spectrum of UV light, in killing during this phase, is one similar to the absorption spectrum of protein. It is possible that the sensitive phase, which shows a nucleic acid type action spectrum, is the period of DNA synthesis. There are good reasons for assuming gene and DNA replication are synonomous, but it would be of great interest to know the precise relationship between the two, for though DNA is a major factor in the 'gene', it must be expressed before any lethal mutations can be observed. The expression of a gene involves several macromolecules other than DNA, and thus the chemical replication of DNA may be entirely different from the phenomena

observed in the ultraviolet light studies.

The results from the work on S. pombe, which have been discussed on the preceding pages, have been summarised in a cell cycle map shown in figure 1. It can be seen that the events that have been observed so far fall into two main groups: those clustered around the time of division and those occurring at about 0.4 of the way through the cycle. The purpose of the results described in this thesis is to add the time of DNA synthesis to this map, and to thereby add to the discussion of the control of synthetic events in the cell cycle.

Can we predict the pattern of DNA synthesis in S. pombe by looking at DNA replication in other organisms? There are reports concerning DNA synthesis in many different organisms, and not surprisingly there is a large variation in the details of these. Even so, common features do emerge.

Before this is attempted one should be clear about the terminology used to describe the DNA synthesis cycle. The one that has been universally adopted is that originally proposed by Howard and Pelc (1953). There are four distinct phases: G1, the presynthetic phase; S, the synthetic phase; G2, the postsynthetic phase and M, which, in their system, represents mitosis and which separates G1 and G2. It is necessary to be careful how M is defined. In higher cells where nuclear division and cell division are normally both parts of the


N.D
C.P
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0
.1
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1.0
X 2 rate of dry mass synthesis
Stepdown — X 2 rate of mRNA synthesis
X 2 amount of TSase
Stepdown — increase in 3H adenine / decrease in 3H uracil incorp
Constant volume
Sensitive to killing by UV & 32P decays — action spectrum of nucleic acid
Log. phase — decrease in rate of RNA synthesis
Resistant to killing by UV & 32P decays — action spectrum of protein
Gene replication — pedigree analysis
X 2 amount of ATCase & OTCase
X 2 rate of synthesis of basal sucrase, acid & alkaline phosphatase
X 2 rate of induced sucrase synthesis
Exponential Synthesis of RNA, Protein & Carbohydrate in exponential growth
N.D
C.P
Div
Div
0
.1
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.4
.5
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.7
.8
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1.0

continuing process of mitosis the question does not arise. But in some simpler organisms, of which S. pombe is an example, where nuclear and cell division are separated by finite and measurable periods of time, this question is important. If, for example, M represents cell fission, and DNA synthesis occurs between nuclear division and fission, then, by definition, the cell would have a long G1 and a very short G2. If, however, in the same situation M is considered to represent nuclear division, then the DNA cycle would be described in a totally different way. There would now be a very short G1 and a long G2. The latter obviously makes more sense because DNA replication is foremost a property of the nucleus. Also the G2 phase is by definition the postsynthetic period, so cells at this stage in the cell cycle should contain the replicated amount of DNA, which would only be the case in the second of the two schemes.

The most striking feature to emerge from the work on the DNA synthesis cycle of various organisms is that in most cases it is periodic; that is to say that most cell types have demonstrable G1, S and G2 phases. This is the case for nearly all mammalian cell types so far studied, both in vivo and in vitro (Cleaver, 1967), plant tissues (Clowes, 1965; V'ant Hof, 1966; Wimber and Quastler, 1963), chicken duodenal epithelium (Cameron, 1964), bullfrog lens epithelium (Reddan and Rothstein, 1966), sea urchin eggs (Hindegardner et al., 1964), Paramecium

(Kimball and Barka, 1959), Tetrahymena (Prescott and Stone, 1967), Euplotes (Prescott, 1966), Amoeba (Prescott and Goldstein, 1967), Physarum (Nygaard et al., 1960) and Saccharomyces (Williamson, 1965). In eukaryotic cells it seems that there is always some degree of periodicity in the DNA synthesis cycle.

This is not the case for the prokaryotic bacterial cells. In these organisms it has been shown by several workers that DNA is synthesised continuously throughout the cycle when the medium on which the bacteria are growing supports rapid growth (e.g. Young and Fitzjames, 1959; Schaechter et al., 1959; Lark, K., 1966; Stonehill and Hutchinson, 1966; Eburle and Lark, 1967; Clarke and Maaløe, 1967). At slow generation times periodicity is developed to give DNA synthesis cycles similar to those of eukaryotic cells.

Though it may be said that eukaryotic cells exhibit periodic synthesis of DNA, there is a large variation in the precise details of the relative lengths of the G1, S and G2 phases depending on the cell type that is being studied. One feature that does emerge as a general rule is that the S phase is a continuous period of synthesis of DNA once initiation has occurred and there are no reports of cells, which have been grown under normal conditions, that show parts of the total synthesis of DNA at different and completely dissociated times in the cell cycle.

There are cells that have no G1 and others that have no G2, though the majority lie somewhere between these two extremes. Robbins and Scharff (1967) have shown the absence of a detectable G1 phase in a cultured strain of chinese hamster cells. Clowes (1965) has shown that within different areas of the root tip of Zea mais there is a large variation in the length of the G1 phase. In the cap initial cells the S period follows immediately after mitosis, whereas in the quiescent zone of the same material the G1 phase constitutes 0.87 of the complete DNA cycle. In sea urchin eggs the S phase follows immediately upon telophase and lasts for 13 minutes in a cycle of one hour. There is certainly no G1 in this system, and it is to some extent open to doubt whether the post S period represents a true G2 or is simply the beginning of mitosis (Hindegardner et al., 1964). The G1 phase is also absent from Amoeba (Prescott and Goldstein, 1967), and the slime mould Physarum (Nygaard et al., 1960). Another example of the absence of G1 is found in the micronucleus of Tetrahymena, but the interesting point here is the fact that the micro- and macronuclei undergo replication of DNA at different stages of the cell cycle (McDonald, 1960; Flickinger, 1967). In this case there is a difference between two nuclei within the same cytoplasm, the micronucleus having no G1, the macronucleus having a G1 equal to about one third of the total cycle.

Other cells have no G2 phase, e.g. Euplotes (Prescott,

1966). Yet others have neither G1 nor G2, for example, grasshopper neuroblasts (Gaulden, 1955), or the sea urchin egg and bacteria that have already been mentioned.

The matter is further complicated by the fact that the precise relationship between the lengths of the DNA synthesis phases is not rigid even within one cell type. Changes in the growth rate brought about by various means have been shown to selectively affect particular phases of the cell cycle. In bacteria it is possible to introduce periodicity into the synthesis of DNA by small increases in the generation time due to the use of different carbon sources in the medium (e.g. Helmstetter, 1967; Clarke and Maaløe, 1967). Lark, C. (1966) has also shown that, at very slow growth rates, there are large portions of the Escherichia coli cell cycle that are devoid of any DNA synthesis. The early work on synchronised bacterial cultures also showed that, under certain conditions, it is possible to observe periodicity in the bacterial DNA synthesis cycle (Barner and Cohen, 1956; Maruyama, 1956; Maruyama and Lark, 1962). All these changes are due to alteration of the growth medium, but an increase in the generation time due to a decrease in the culture temperature seems to have little effect on the position of the S phase in bacterial cell cycles (Schaechter et al., 1958).

If periodicity is introduced into the bacterial DNA synthesis cycle it is difficult to relate to the concepts of

G1, S and G2 as applied to higher cells. When the cells were grown with generation times faster than 60 minutes, the observed periodicity was in the rate of synthesis and not in the presence or absence of synthesis (Clarke and Maaløe, 1967; Lark, K., 1966; Helmstetter, 1967; Helmstetter and Cooper, 1968). At generation times greater than 120 minutes events were clearer and it seems that the periodicity under these circumstances was prior to the synthesis of DNA and thus might be considered as an extended G1 phase (Lark, C., 1966). There is some disagreement over what happens at the faster growth rates (Lark, K., 1966; Cooper and Helmstetter, 1968), which could be due to the differences between the strains of E. coli used in these studies. The results of Helmstetter and Cooper (1968) show that the time, relative to the following cell division, at which a new round of replication is started, and the time that it takes to complete the replication of one chromosome, are constant over a wide range of generation times. In this model it is the pre-initiation phase that is increasing, which could be considered equivalent to G1 of higher cells. It is harder to relate G1 to the model proposed by Lark, K. (1966). Nevertheless, because the amount of DNA/cell decreases as the generation time increases (Lark, C., 1966), there must be delay to the initiation of some rounds of DNA synthesis, which could be considered as an extension to G1.

In higher cells the G1 phase also appears to be the most

sensitive to changes in the environment. Changes in the composition or pH of the growth medium have been shown to selectively increase the G1 phase in mammalian cells (Defendi and Manson, 1963). Sisken *et al.* (1965) have shown that temperature changes can have a similar effect on tissue culture cells, and Sherman *et al.* (1961) suggested that the long G1 in mouse epidermal cells is a result of the fact that they are subject to lower than normal temperatures. Even without experimentally altering the environmental conditions, it has been shown that it is the G1 that is the most variable, not only between different cell types of the same organism, but also between different cells of the same type (Terasima and Tolmach, 1963; Sisken and Morasca, 1965; Cameron, 1964).

It is thus difficult to make predictions on the basis of the patterns of DNA synthesis exhibited by other organisms, except, perhaps, that one might expect that the cell cycle of *S. pombe* would have detectable G1, S and G2 phases. It might also be expected to show close similarities to the DNA cycle in *Saccharomyces cerevisiae*. In this organism the synthesis of DNA is sharply periodic and the S phase occurs about midway between two nuclear divisions, just after the buds appear (Williamson, 1966).

We should now turn to the question of how one might investigate the DNA synthesis cycle in *S. pombe*. It is not possible to get an answer by the methods used so far in other biological

systems. Unfortunately ^{3}H-thymidine is incorporated into RNA in S. pombe and, even after ribonuclease digestion, the label is distributed throughout the cytoplasm. The method of labelling with ^{3}H-adenine, followed by removal of the RNA, used by Williamson (1965) for S. cerevisiae, again yields an even distribution of grains over the cytoplasm. The treatment necessary to remove the label over the cytoplasm results in the complete loss of label or the destruction of the cells (see chapter 5).

Another approach to this problem has been the estimation of the DNA content of individual nuclei by microspectrophotometric techniques. Again this is not possible in S. pombe because of the low absolute amount of DNA, and also the high RNA:DNA ratio.

A third approach has been the use of synchronous cultures (e.g. Young and Fitzjames, 1959; Abbo and Pardee, 1959), coupled with incorporation of labelled precursors or bulk biochemical estimations of DNA content. At the time that this work was started a satisfactory method for obtaining synchronous cultures of S. pombe had yet to be developed, although this was achieved later by Mitchison and Vincent (1965). It is therefore necessary to attack the problem from a different and more indirect approach.

The reasoning behind the experiments is essentially

similar to that in the experiments of Yoshikawa and Sueoka (1963). At any given point during exponential random growth the culture will consist of cells at all stages of the cell cycle. There will be twice as many young cells as there will be cells just ready to divide, and the number of cells that are between 0.0 and any stage in the cycle is given by the relationship

$$\frac{d}{T} = \frac{\log_e(1-\frac{a}{2})}{\log_e 0.5}$$ (see Appendix 4) - equation (1)

where d is the stage in the cell cycle (T), and a is the proportion of cells between 0.0 and d. It follows that 1 - a is the proportion of cells that have passed d. Thus if an estimate of the mean DNA content of the culture is made it will be composed of some in the unreplicated state, some in the replicated state and others in the process of replication. If the amount of DNA present in a G1 cell is known it is then possible to calculate the stage in the cycle that the mid-point of replication occurs. This will only show the point at which half the DNA is synthesised, and not the length of synthesis; if the synthetic period is long in relation to the whole cycle the estimated position becomes less accurate. It also assumes a constant rate of synthesis during the synthetic period.

In order to make use of the above formula it is necessary to know two things. Firstly the mean DNA content of cells during random exponential growth, and secondly the DNA content

of the replicated nucleus. There are two phases in the growth cycle at which the cells might be expected to contain single unreplicated nuclei: during stationary phase and in the ascospore stage. There are indications that suggest the division processes take precedence over the other synthetic events of the cell, such that stationary phase cells would contain G1 nuclei (Swann, 1957). It has, in fact, been shown that many cells do stop growing in G1, coming to rest with the unreplicated amount of DNA per nucleus (e.g. Williamson and Scopes, 1961; Kimball and Vogt-Kohne, 1961; Stonehill and Hutchinson, 1966). There are exceptions, for example, Tetrahymena pyriformis stops growth in G2 (McDonald, 1958), and the G2 population of mouse ear epidermal cells might be included in this category, though there is some doubt as to whether growth is arrested or has been greatly slowed down (Gelfant, 1962).

Provided the stationary phase population is homogeneous with respect to the point of arrest in the cycle it would not really matter whether the cells were in G1 or G2. If it was G1 then the mean DNA content in log. phase would be higher than that in stationary, whereas if it was G2 then the log. phase would be lower. Difficulties arise if the stationary phase population consists of a mixture of G1 and G2 cells, and it is for this reason that it is necessary to check the G1 nuclear value against the DNA content of ascospores. If the stationary phase is a homogeneous population of G1 cells it should show the

same DNA content per cell as the spores, because the strain of S. pombe used in this study is haploid for the majority of the growth cycle; the only phase in which it is diploid is after conjugation, when the zygote is formed prior to the production of ascospores (Leupold, 1956).

Thus from careful measurements of the mean DNA content of cells during the exponential, and into the stationary, phases of growth, and also in the ascospore stage, some information could be obtained about the DNA synthesis cycle in S. pombe. One drawback of this method of analysis is the fact that all the estimates are of total DNA, and no account can be taken of the possible presence of DNA in cytoplasmic organelles. That such DNA could be present in effective quantities became apparent after the bulk of the work described in chapters 2 and 3 had been carried out. Amongst the reports of mitochondrial DNA the most relevant to this topic is the fact that S. cerevisiae contains 20% of its total DNA in the mitochondria in stationary phase, but that during the log. phase this is reduced to 1 or 2%.(Moustacchi and Williamson, 1966). A mitochondrial DNA contribution of this magnitude would introduce serious errors into the assumptions of the scheme proposed above. Because of this, the proportion of mitochondrial DNA at various stages of the growth cycle was investigated at a later date.

There are grounds for expecting the other phase - the lag

phase - of the normal growth cycle to yield further information about the DNA synthesis cycle. The lag phase is used here to describe the period during which the culture increases in mass, but not in cell number. It begins immediately stationary cells are inoculated into fresh medium, and continues until the first wave of cell division is observed. Several organisms will grow out of the stationary phase as a synchronously dividing population, when inoculated into fresh medium (e.g. Streptococcus faecalis, Stonehill and Hutchinson, 1966; Bacillus subtilis, Masters and Donachie, 1966, and Cutler and Evans, 1966; and, with additional selection procedures, S. cerevisiae, Williamson and Scopes, 1960). Though, as will be shown later, S. pombe will not divide synchronously in such a situation, there is an initial semi-synchronous burst of division. It is possible that the lag phase represents an extended, and no doubt special, cell cycle. By following the course of DNA synthesis during the lag phase more information about the DNA synthesis cycle should emerge.

This is the rationale behind the experiments described in chapter 2; such a system could yield very little information, but, as we shall see, in S. pombe this approach was rewarding. It has one advantage over the other ways of tackling this problem in that it overcomes the formal objections to the synchronous culture - the relationship between the synchronous and the random, and supposedly normal, culture - and the

difficulties involved in autoradiography. The latter is dependent on, amongst other things, a constant rate of uptake of label into the acid soluble pool and also a constant ratio between the endogenous and exogenous supplies of the tracer (discussed in Cleaver, 1967, and Mitchison, 1963).

Chapter 2

The Normal Growth Cycle

Materials and Methods

The Organism

The fission yeast, Schizosaccharomyces pombe (Linder) N.C.Y.C. 132, was used throughout these studies. The mean generation time, when in exponential growth in Edinburgh Minimal Medium (EMM 1), is 2 hours 20 minutes at 32°C. During this phase of growth the cells increase in length only, being between 6 and 20 μ long and 3.5 μ in diameter, whereas in stationary phase the cells are uniformly small, with only small variations in both length and diameter.

The homothallic sporulating strain, H 90, of S. pombe was used for the estimations of the DNA content of ascospores.

Both these strains of S. pombe are haploid for the majority of their growth cycle. The only stage at which they are genetically diploid is during sporulation, when fusion of the nuclei from the conjugating cells results in the formation of a diploid zygote. The zygote nucleus then undergoes two divisions to form four haploid ascospores (Leupold, 1956).

Growth Media and Conditions

Cultures were grown on EMM 1 (see appendix 1) at 32°C

(± 0.5) in either a water bath or a warm room. Stock cultures were maintained in 10 ml. of medium in McCartney bottles. Normal sterile precautions were observed at all times. The medium was autoclaved for 10 minutes at a pressure of 10 lbs./in.^{2} in all cases. Inoculations were carried out with pipettes which had been sterilised in an oven for 3 hours at 130°C. However, as the medium is acidic (starting pH = 5.5), it is unfavourable for bacterial growth, and contamination of cultures is rare.

For the log./stationary phase experiments the medium was inoculated on the day before the experiment with a small quantity of stationary phase culture at a cell concentration of approximately 25 x 10^6 cells/ml., such that the batch culture would be in early log. phase (1 - 2 x 10^6 cells/ml.) on the morning of the experiment. Samples were removed, and the cells harvested by means of centrifugation, throughout the growth of the culture into stationary phase. After harvesting the samples were washed three times in distilled water, and stored in the deep freeze until all the samples from the culture were ready for extraction and assay. The size of sample was adjusted throughout the experiments so that each would contain 5 - 20 µg. DNA. Culture growth was monitored periodically by cell counts and optical density measurements at 595 mµ in an Unicam S.P. 500 spectrophotometer.

For the lag phase experiments 1.5 litres of culture were

grown to stationary phase and left for 3 days. Before the cells were harvested for the experiment, samples were removed to check the stationary phase DNA content. After harvesting, by centrifugation, the cell pellet was quickly raised in 50 ml. of fresh pre-warmed medium at 32°C, and the cell suspension inoculated into 5 litres of fresh medium. Samples were taken at 5, 15, 30, 45 and 60 minutes after inoculation, and thereafter at 30 minute intervals. These experiments were carried on for about 8 hours, by which time the culture had almost doubled in cell number. The samples, as in the previous experiments, were collected, washed three times in distilled water and stored in the deep freeze until all the samples could be extracted and assayed at the same time. Culture growth was monitored by cell counts, cell plates and, occasionally, optical density.

H 90 S. pombe was grown on "Oxoid" Malt Extract Agar (MEA) slopes in petri dishes at 32°C for 7 days, after which they were transferred to 4°C for a further 7 days. This treatment resulted in the highest yield of ascospores, which in most experiments varied between 80 and 90%. Stock cultures were maintained on MEA, subculturing from colonies that contained spores. H 90 shows a constant tendency to revert to the wild type heterothallic mating type, and, if the percentage of spores is to be maintained at a maximum, it is necessary to subculture from colonies that are still homothallic. These

were identified by inverting the petri dish over a container in which iodine crystals were being gently heated. The spore-containing colonies were stained brown by the iodine vapour.

Colonies showing a positive iodine reaction were picked off with a bacteriological loop and streaked out on fresh MEA slopes and taken through the sporulation treatment. After fourteen days the spores were washed off with distilled water, washed three times in distilled water and layered on top of a sucrose gradient for separation (see next paragraph). After separation the spore sample was counted and split up into separate samples, so that each contained between 5 and 20 ug. DNA.

Separation of Spores

The procedure is essentially similar to that used for making synchronous cultures, which is described in more detail in the next chapter. The spore and stationary phase cell mixture was layered on a 10 - 40% sucrose gradient and centrifuged for 10 minutes at 1500 r.p.m. in an M.S.E. Mistral 4L centrifuge. The cells and spores moved down the gradient at slightly different rates due to their different size. The spores tended to move slower resulting in the top layer of cells in the gradient being enriched with spores. To get an essentially cell-free spore suspension it was necessary to recycle this upper layer. This was done for a few estimates of

DNA, but the yield after such treatment is less than 0.5% of the original sample. Because of this, in other experiments only one gradient separation was made, but the proportion of stationary phase cells was counted and an allowance made for the contribution made by these to the DNA in the final extract, though in all experiments this was very small.

Cell Counting

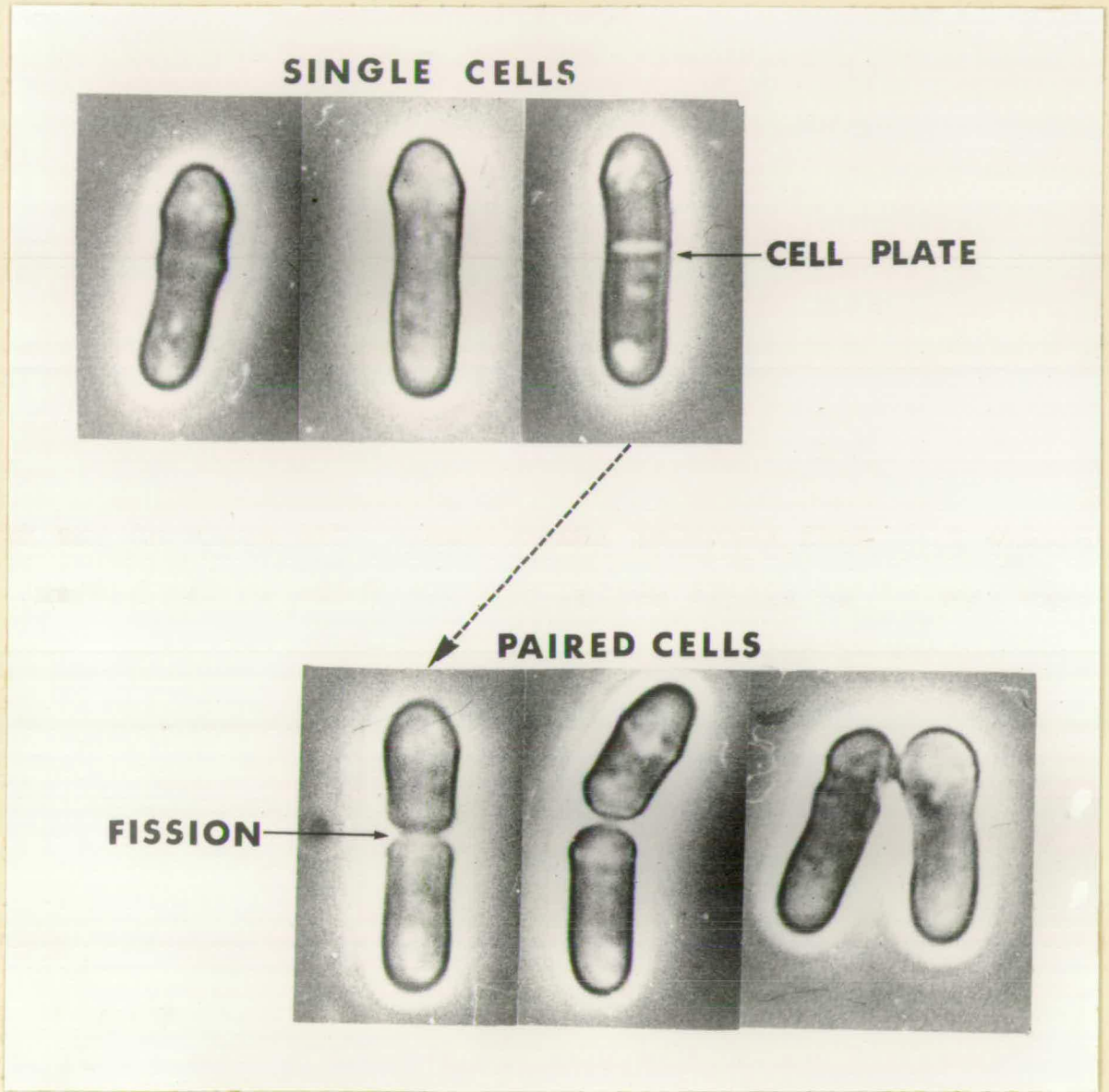
Initially cell counting was done by means of an Improved Neubauer Haemocytometer, depth 0.1 mm. Cells were considered to have divided once fission could be observed (see figure 2). Counting samples were diluted so that approximately 500 cells would occupy each counting chamber of the Haemocytometer, giving a final count of between 500 and 1000. This gave a counting error of around 5%.

It was not always possible to count samples at the time that they were withdrawn from the culture. In these cases the counting samples were either stored in the cold or they were diluted with an equal quantity of 4% formalin. If the samples could be counted within 24 hours the former method was used, but if the samples were required to be kept for longer periods of time the formalin was used.

Coulter Counting

In later experiments cell counting was done using a

Figure 2. Cell counting convention. The cells on the top were counted as single cells, whereas those on the bottom were scored as two cells.


SINGLE CELLS
CELL PLATE
PAIRED CELLS
FISSION

Coulter Counter Model B, with a 70 μ orifice tube. Cell clumps and pairs were separated by ultrasonic disintegration in an M.S.E. 100 watt Ultrasonic Disintegrator, fitted with a $\frac{3}{8}$" titanium probe, for 90 seconds at a frequency of 20 kc/sec. and an amplitude of 2 μ. This treatment resulted in 98% of the cells existing as single cells, the remainder being in pairs.

Cell Plate Index

The cell plate index is a measure of the number of cells dividing, and is expressed as the percentage of cells showing cell plates. 0.5 ml. samples were withdrawn from the culture, spread on a microscope slide, and allowed to dry on a warm plate. The slide was then washed to remove excess salts and glucose, negatively stained with waterproof black ink (10% Pelican Ink, 0.1% formalin and 4 drops Byprox in distilled water), dried and stained with 0.25% crystal violet.

Nuclear Staining

Samples were removed from the culture and dried down on a clean microscope slide. The method used for the fixing and staining of cells was a modification of that used by Ganesan and Swaminathan (1958). The cells were fixed in acetic:alcohol (1:4), rinsed in chloroform and dried. RNA was extracted by digestion for 3 hours in ribonuclease (RNase) solution. Crystalline pancreatic RNase (Sigma) was used at a concentration of 250 μg./ml. in 0.05 M Tris/HCl buffer, pH = 7.6, after it had

been heated in a boiling water bath for 10 minutes. After washing in running tap water the slides were immersed in 4% Giemsa (Gurr) in phosphate buffer, pH = 7.0, for 16 hours. The slides were differentiated by moving them about in a very dilute solution of acetic acid, pH around 4.5, and observing them under the microscope every 2 minutes. When the cytoplasm was sufficiently de-stained the slides were mounted in a drop of dilute stain, and the coverslip sealed with vaseline and wax. The quality of staining varied considerably between batches of slides, one of the major causes being the variation in the efficiency of RNA extraction from the cytoplasm. In cells where the nuclei were clearly visible they appeared as darkly stained bodies, but there was always a percentage of cells in which they could not be identified, due either to overstaining of the cytoplasm or lack of stain in the nuclei.

Estimation of DNA

Cells were harvested by centrifugation and washed three times in distilled water. DNA extraction was by the Schneider (1945) procedure, with slight modifications (Hutchinson and Munro, 1961). The cells were suspended in 0.5 M perchloric acid (PCA) for 30 minutes at 0°C, and washed twice in cold 0.5 M PCA. After the second washing the tubes were dried inside with a Kleenex tissue to remove as much liquid from above the cell pellet as possible. The cells were then extracted with 0.5 M PCA at 70°C for 20 minutes, centrifuged,

the supernatant being removed and retained, and re-extracted with 0.5 M PCA at 70°C. The supernatants from the two extractions were pooled and the purine-bound deoxyribose assayed by the diphenylamine method.

Initially the standard Burton (1956) diphenylamine method was used to estimate the DNA content of the extracts. 0.2 ml. of the extract was incubated with 0.4 ml. of the diphenylamine reagent (2% diphenylamine, 1.5% concentrated Analar H_2SO_4, and 0.1 ml. aqueous acetaldehyde, 16 mg./ml., in glacial acetic acid) for 16 hours, and the absorption at 595 mμ was measured in a Unicam S.P. 500 spectrophotometer. During the course of the work this technique was modified as follows: 0.2 ml. of the extract was incubated with 0.4 ml. of the diphenylamine reagent (4% diphenylamine in glacial acetic acid), plus 0.01 ml. of aqueous acetaldehyde (1.6 mg./ml.), overnight for 16 hours and the absorption at 595 - 700 mμ read in the spectrophotometer. This modification followed a paper by Giles and Myers (1965) and increases the sensitivity by a factor of 50%. There is a tendency in both these methods, but more so in the latter, for turbidity to develop during incubation. The effect of this can be eliminated by centrifuging the coloured solution before reading in the spectrophotometer, and then subtracting the absorption at 700 mμ from that at 595 mμ. This gives low blank readings and a linear calibration curve for concentrations of deoxyadenosine up to 20 μg./ml. All assays

were done in triplicate, the mean absorption being used to determine the concentration of purine-bound deoxyribose from a calibration curve. These curves were constructed using solutions of deoxyadenosine in 0.5 M PCA, one such curve accompanying each set of assays.

The use of deoxyadenosine as the standard requires that a correction be made in order to express the results in terms of grams of DNA/ml. Assuming that the purine-bound deoxyribose accounts for 50% of the bases in the DNA it is necessary to multiply the value in terms of grams of deoxyadenosine/ml. by 2.605 to express it in terms of grams of DNA/ml.

The Sneider method of extracting the DNA was found to be inadequate for the spores. As a result two methods were tried to obtain values for the amount of DNA in spores. The first was to break the spores by ultrasonic disintegration, and to assay DNA in the acid precipitable fraction of the sonicate. This was not considered ideal because it is known that ultrasonics also break up DNA, and there was the possibility that the breakdown products would be too small to be acid precipitable, though no diphenylamine material could be shown to be present in the cold PCA soluble fraction. A second method was used as a check. The spores were digested in 1 N NaOH for 6 hours at 32°C. Cold 1 N PCA was then added to make the final solution 0.5 N PCA and the cold acid insoluble fraction removed by centrifugation at 12,000 r.p.m. for 10 minutes. The

precipitate was then raised in 0.5 N PCA, heated at 70°C for 20 minutes and assayed for DNA as described previously. In both these methods assays for diphenylamine positive material were made on the acid soluble supernatants, but no detectable amounts of this material were ever found.

Although the diphenylamine reagent is supposed to be specific for purine-bound deoxyribose residues, there have been reports of interference from other cellular components, especially in plants (Lee, 1963). Deoxyribonuclease (DNase) controls were initially performed on samples from different stages of the growth cycle to see if any interfering substances were present in the hot PCA extracts. Unfortunately, DNase will not enter cold acid extracted cells, so it is necessary to perform an alkaline hydrolysis before the DNase treatment. The method was as follows: the cells were harvested, washed in distilled water, cold acid extracted in 0.5 N PCA, and washed twice in cold 0.5 N PCA. They were then hydrolysed in 1 N KOH for 6 hours at 32°C. 1.0 N PCA was added in the cold to make a final solution of 0.5 N PCA and the precipitate collected by centrifugation at 12,000 r.p.m. for 10 minutes. The precipitate was washed twice in cold 0.05 M tris/HCl buffer (pH = 7.5) and was finally raised in 0.05 M tris/HCl buffer containing 250 µg. DNase/ml. and incubated at 37°C for 5 hours. An equal volume of cold 1.0 N PCA was added, and the precipitate collected by centrifuging at 12,000 r.p.m. for 10 minutes. The supernatant was removed to another tube and the precipitate

was raised in 0.5 N PCA, both being heated at 70°C for 20 minutes. The supernatant and the hot acid extract of the precipitate were then reacted with the diphenylamine reagent. In all cases all the diphenylamine positive material was found in the DNase digested/acid soluble fraction, confirming that the method was measuring DNase sensitive substances. It is possible that some interfering substances were removed during the alkaline hydrolysis, but in all cases there was quantitative recovery of diphenylamine positive material in the DNase digested fraction.

Results

The Exponential and Stationary Phases of Growth

The results of one experiment are shown in figures 3 and 4, in which the growth of the culture was followed in terms of cell concentration, optical density and DNA content of samples. It can be seen that the increase in cell numbers is exponential up to about 1×10^7 cells/ml., whereas the optical density increase begins to fall off at around $3 - 4 \times 10^6$ cells/ml. Optical density is in part a measure of cell mass and this falling off in the rate of increase in optical density therefore seems to reflect a decrease in the rate of synthesis of mass once the cells have reached this cell concentration. It is well correlated with the observation that the cells become

Figure 3. DNA per ml. (solid circles, solid line), cell concentration (solid circles, dotted line) and optical density (open circles) in a randomly growing culture.

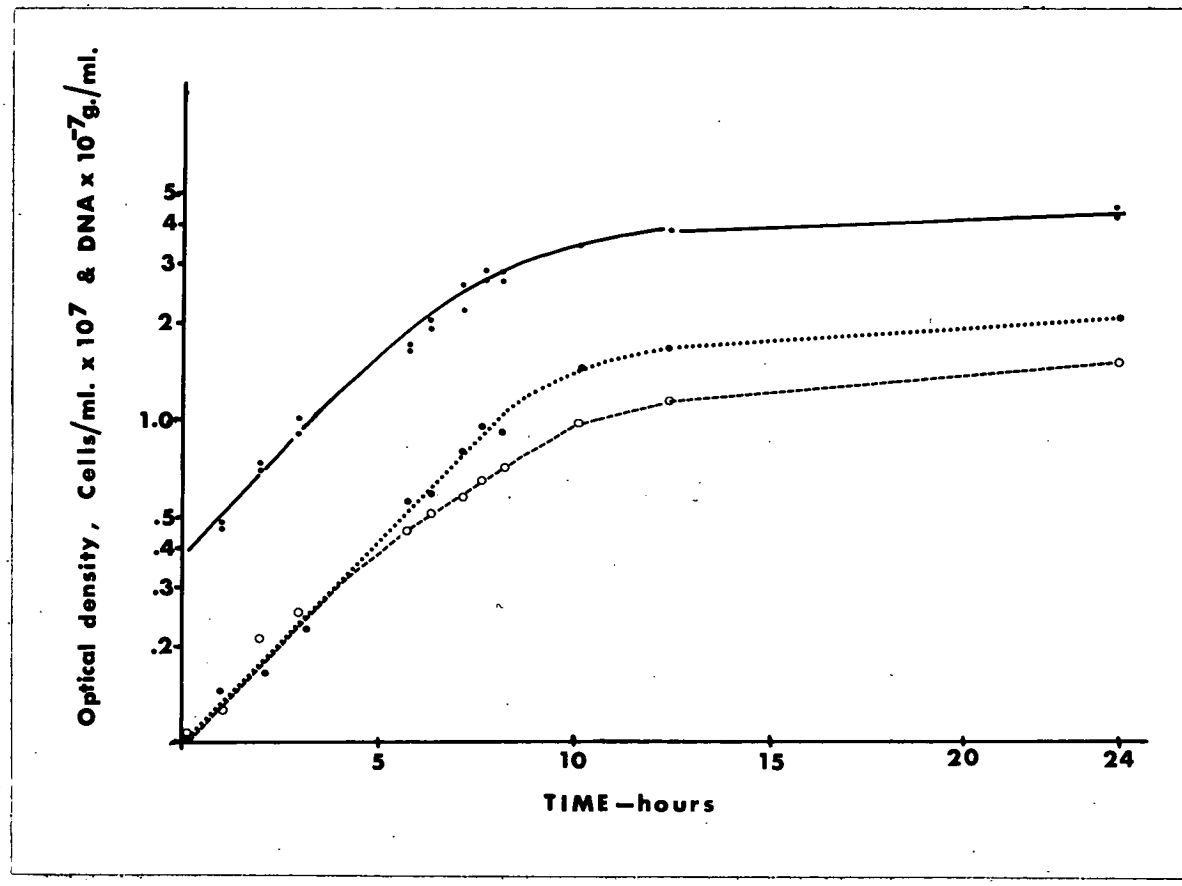
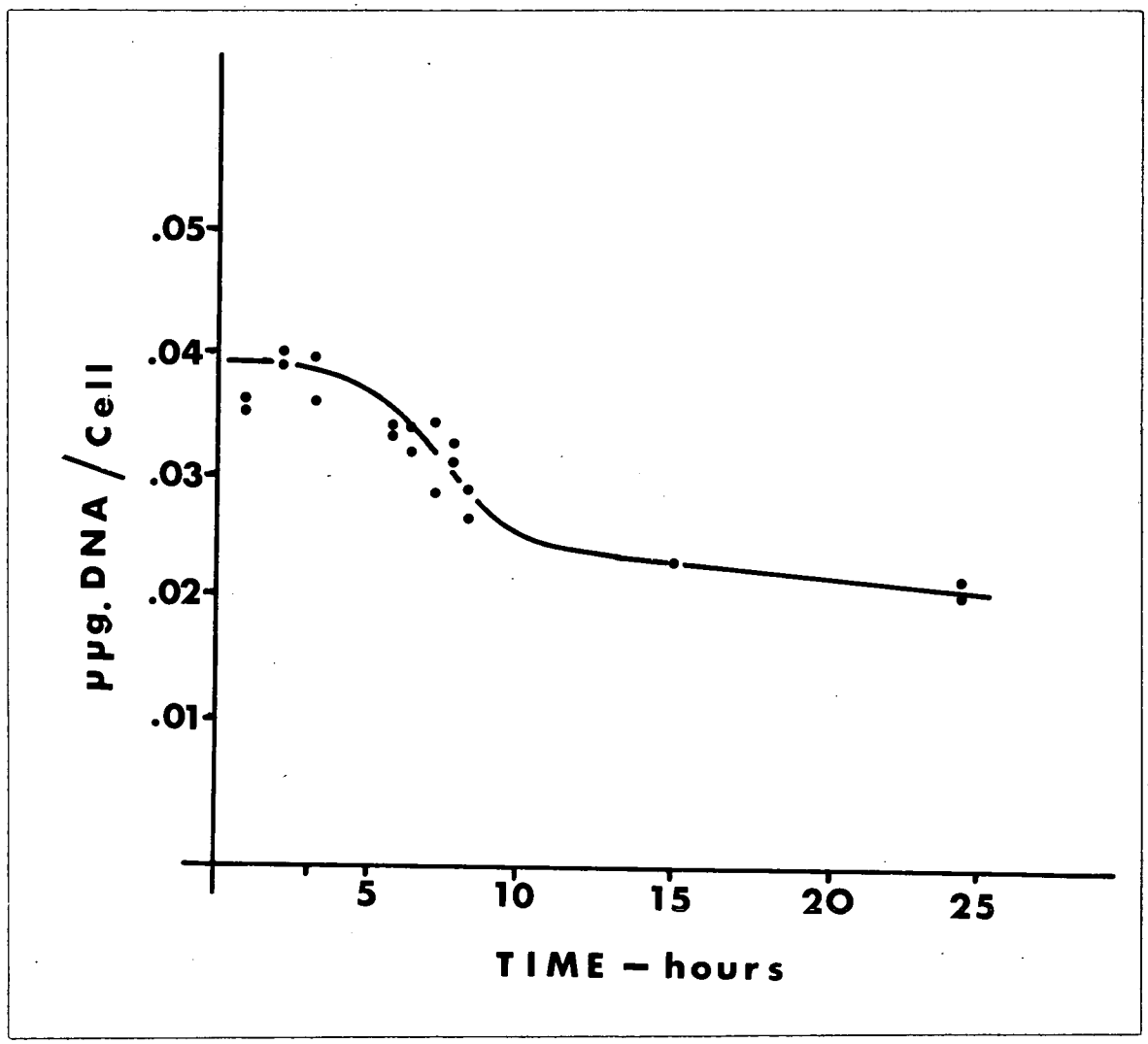

Optical density, Cells/ml. x 10^7 & DNA x 10^-7g./ml.
5
4
3
2
1.0
.5
.4
.3
.2
5
10
15
20
24
TIME—hours

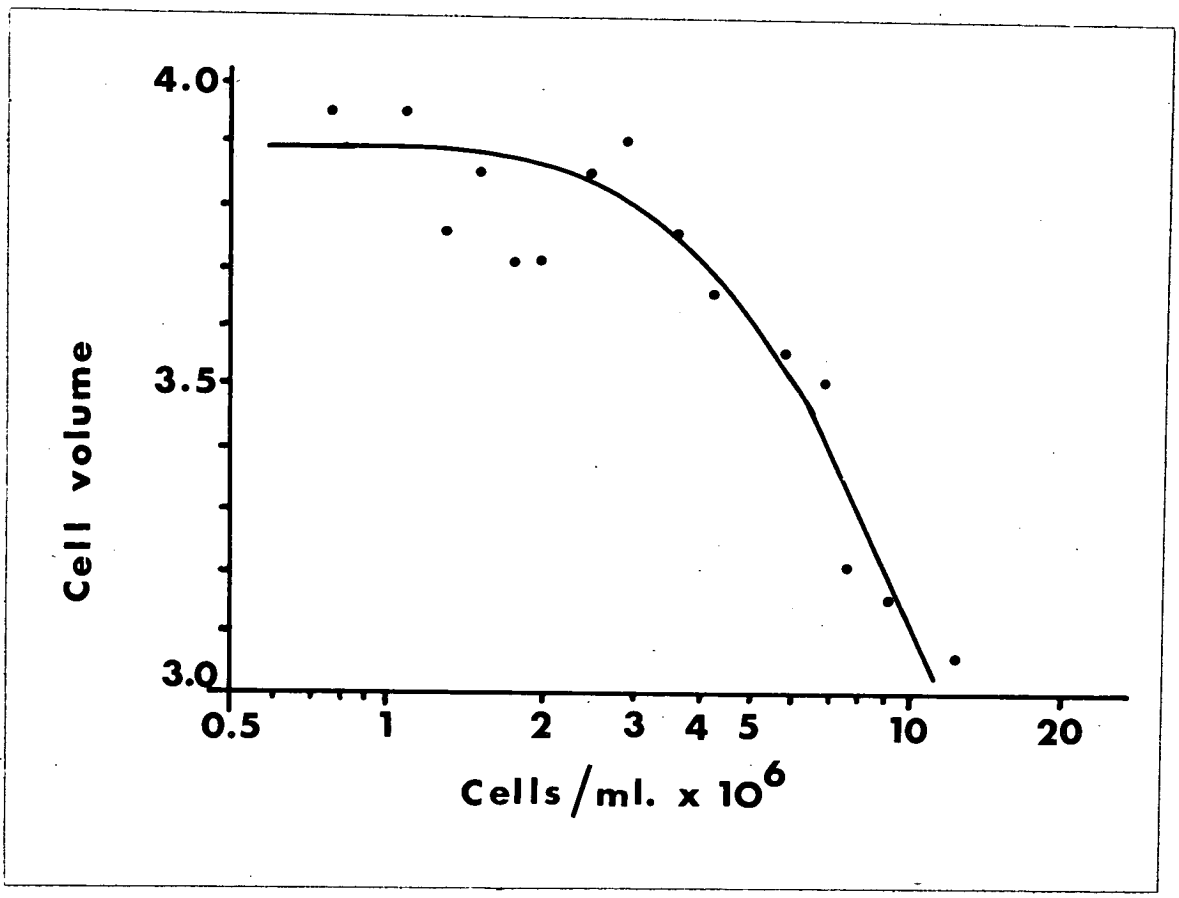
Figure 4. Changes in the amount of DNA per cell with age in a randomly growing culture.


.05
.04
.03
.02
.01
μμg. DNA / Cell
5
10
15
20
25
TIME — hours

smaller as log. phase progresses beyond this point (see figure 5 and Johnson, 1968) and that the rate of total RNA synthesis begins to fall at a cell concentration of 3 x 10^6 cells/ml. (Stebbing, pers. commun.). The curve of the increase of DNA/ml. is of similar shape to that of optical density; although it is not immediately apparent, it shows a decrease in the rate of DNA synthesis after the cells have reached a cell concentration of 5 x 10^6 cells/ml. This becomes more obvious if the mean DNA content/cell is plotted as a function of time (figure 4). In this form it can clearly be seen that the mean DNA content of cells begins to fall between 4 and 6 hours in this experiment, and continues to fall until the cells enter stationary phase.

In order to compare the results of different experiments the results should be expressed as the DNA content/cell as a function of the cell concentration. The combined results of several experiments have been plotted in this way in figure 6. The DNA values have been split into eight groups which are designated by the parallel broken lines. The means of the DNA values within each group, together with the standard errors of the means, are also shown. From these data it is clear that the cells are no longer in balanced growth after the culture has reached 5 x 10^6 cells/ml., the stage at which the DNA content of cells begins to drop.

If the log. phase is considered to last up to a cell concentration of 5 x 10^6 cells/ml. the mean DNA content of log.


4.0
3.5
3.0
Cell volume
0.5
1
2
3
4
5
10
20
Cells/ml. x 10^6

phase cells is 0.0376 µµg/cell (S.E. = 0.0005). Similarly, if cells are considered to be in stationary phase 24 hours after there is no further increase in the number of cells/ml., the mean DNA content of these is 0.0199 µµg./cell. Between these two phases of the growth cycle the mean DNA content/cell is halved over two doublings of the cell number.

Spores

The results of 13 estimations of the DNA content are also shown on figure 6 so that a comparison can be made between the mean DNA content of spores and that of stationary phase cells. That they are different is clear from figure 6; the difference between 0.0199 µµg. DNA/stationary phase cell and 0.0146 µµg. DNA/spore is significant at the 0.1% level.

Reinoculation Experiments

The results from one experiment are shown in figure 7. It can be seen that there is no sign of division in the culture until 4½ hours after inoculation. There is first a rise in the cell plate index, which is closely followed by the increase in cell numbers. The curve of DNA/ml. of culture is more complex. Immediately after the cells are suspended in fresh medium there is a rise in the DNA content of the culture. After an hour it remains constant until cell plates first appear in the culture. At this point it shows a sharp rise, but eventually slows down to show a rate of increase similar to

Figure 6. Changes in the DNA content of cells with increasing cell concentration in randomly growing cultures, and of spores, of S. pombe. The solid circles represent the individual means of each estimate in triplicate, and the open circles represent the means of groups of estimates. The bars show the standard errors of the means and the parallel broken lines show how the estimates have been grouped to obtain the means.

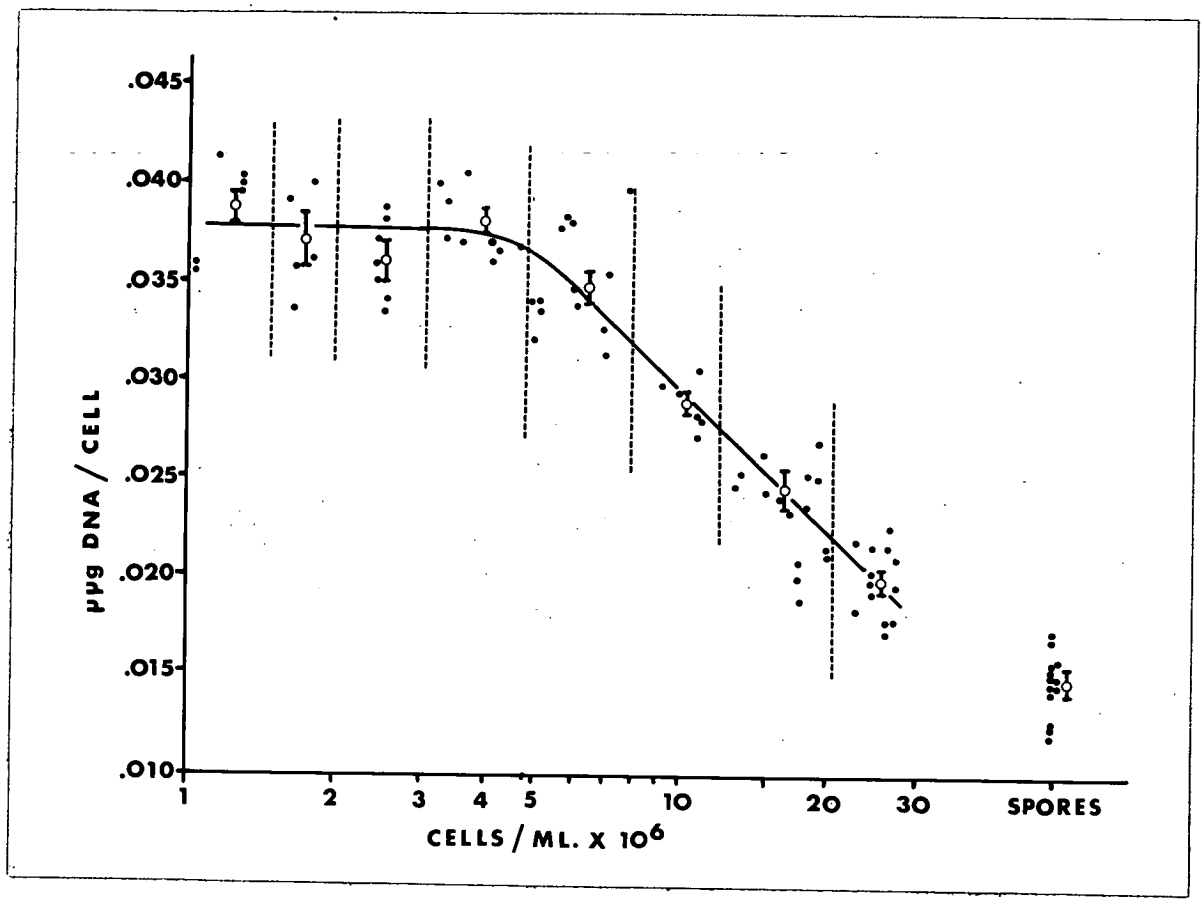
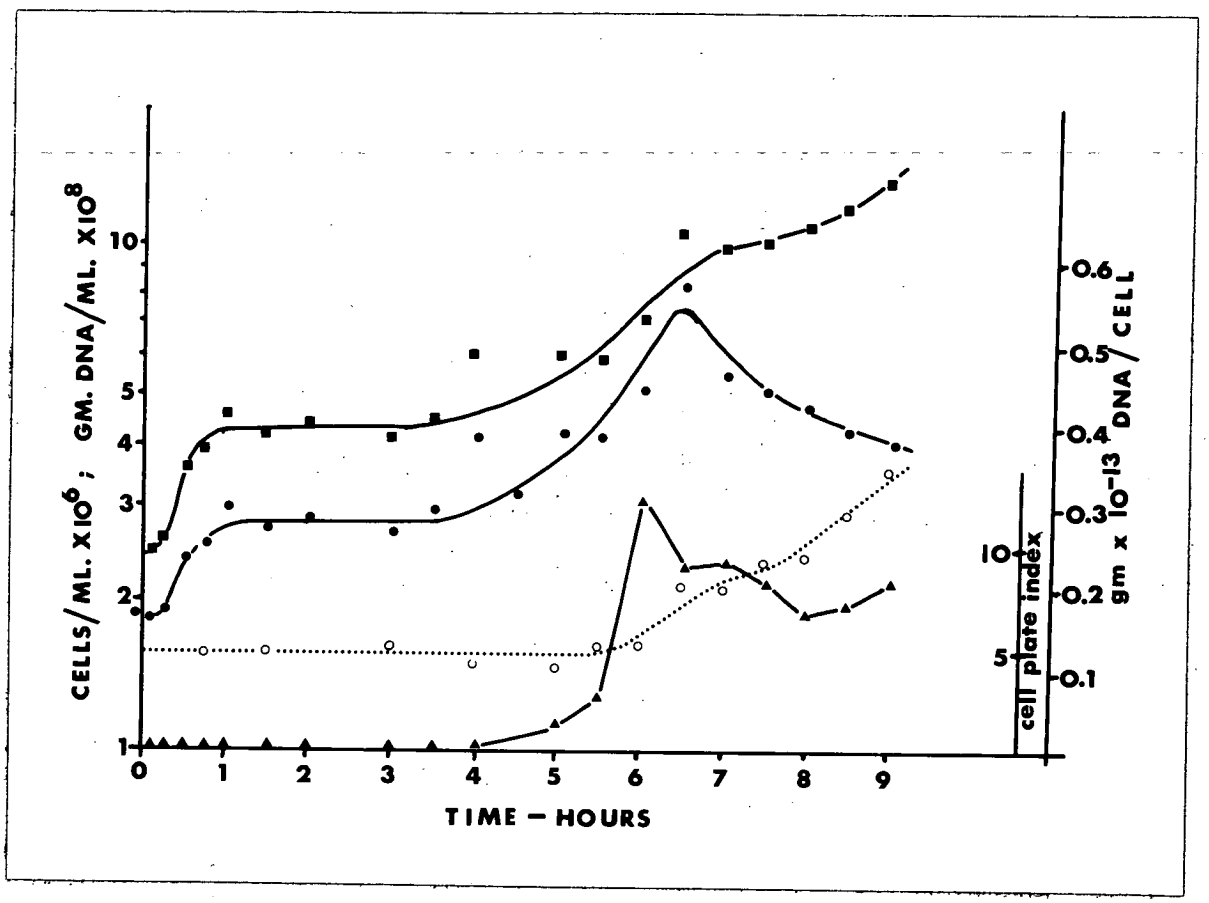

.045
.040
.035
.030
.025
.020
.015
.010
μμg DNA / CELL
1
2
3
4
5
10
20
30
SPORES
CELLS / ML. X 10⁶

Figure 7. DNA per ml. (closed squares), DNA per cell (circles), cell concentration (open circles) and cell plate index (triangles) in a lag phase culture in EMM 1 at 32°C.

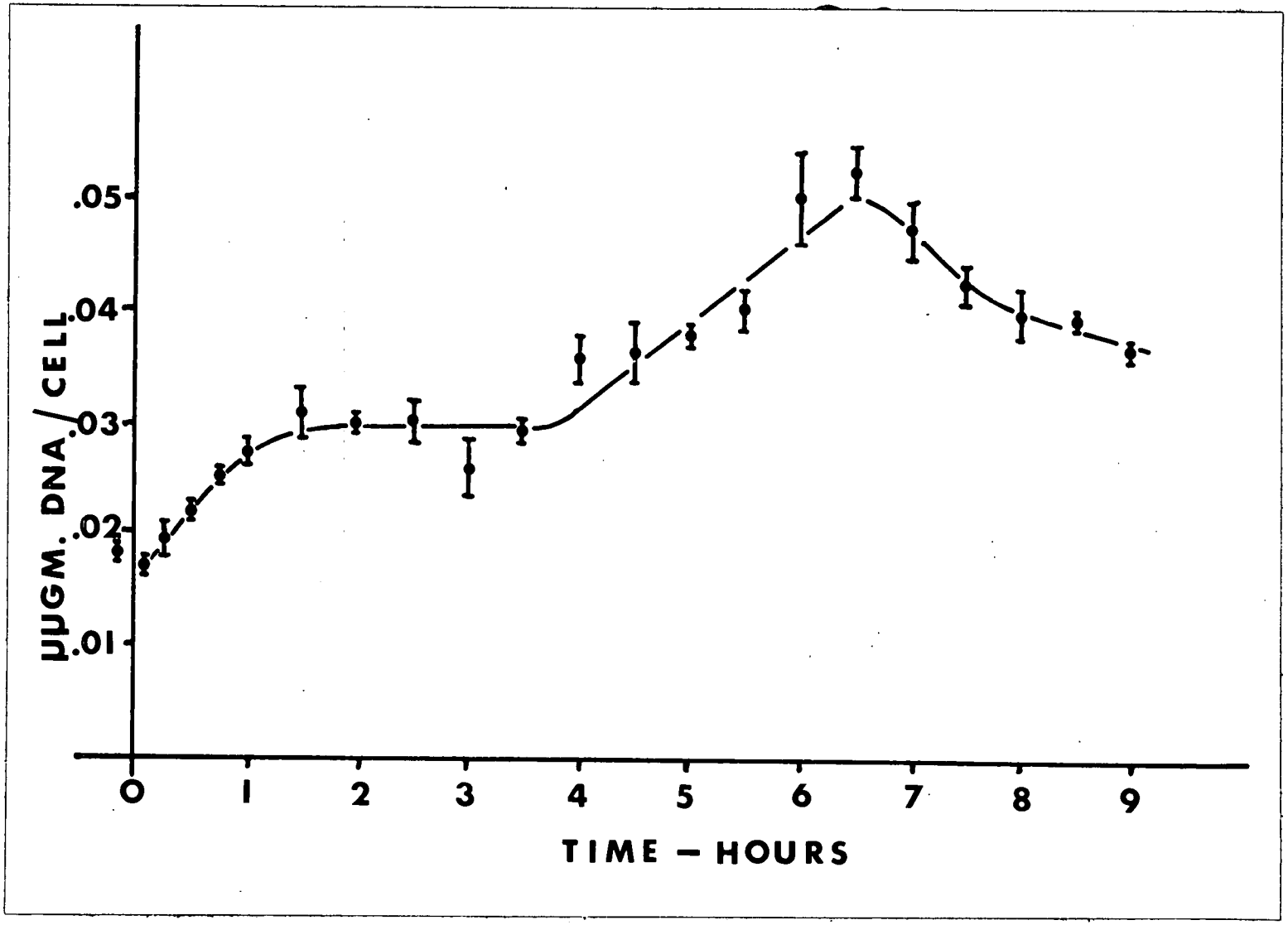

CELLS/ML. X10^6 ; GM. DNA/ML. X10^8
10
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
TIME — HOURS
cell plate index
10
5
gm x 10^-13 DNA/CELL
0.6
0.5
0.4
0.3
0.2
0.1

that of the increase in cell numbers. It is not possible to establish parallel curves of these two parameters because of the necessity to inoculate the medium at a high cell concentration. Thus, at these concentrations, the cells have reached the equivalent of the critical point in log. phase after the first divisions and balanced growth is never established. The results of this experiment, and two others, have been plotted in figure 8, which shows the changes in the mean DNA content/cell as a function of time. The points represent the means of the three experiments, and the bars the standard errors of these means. The line between the points has been drawn by eye, but the exact shape of the curve is not important. The points that emerge from this experiment are as follows. The DNA content of cells before and 5 minutes after inoculation is characteristic of stationary phase. Thereafter, it quickly rises to a value of 0.0292 µµg. DNA/cell and remains constant at this value for a further 2½ hours. At the time that the cell plates first appear it begins to rise to a peak of 0.050 µµg. DNA/cell. After this it decreases due to the increase in the cell number which at this stage is more rapid than the increase in DNA.

Giemsa Staining of Nuclei

The relationship that will be used to position the mid-point of DNA synthesis in the cell cycle assumes an exponential distribution of cells between the beginning and end of the

Figure 8. DNA per cell in lag phase cultures in EMM 1 at 32°C. The solid circles are the means of three experiments and the bars show the standard errors of the means.


.05
.04
.03
.02
.01
μμGM. DNA / CELL
0
1
2
3
4
5
6
7
8
9
TIME – HOURS

cycle. It was considered desirable to check the position of two other events, nuclear division and cell plate formation, that are good cell cycle markers, using the same method. From slides, prepared by Giemsa or indian ink/crystal violet staining, it was possible to assess the proportion of cells which had either two nuclei or a cell plate. From this data it was possible to calculate the stage in the cell cycle that these events occur. Table 1 shows the results of such an analysis at different stages during the phase of exponential increase in cell numbers. It can be seen that in most samples nuclear division takes place at about 0.75 of the cell cycle, and that cell plate formation begins at 0.86. This is in agreement with previous estimates of their positions in the cell cycle using more direct techniques (Mitchison, 1957). It appears that, for relatively instantaneous events at least, this method gives a fair estimate of the position of such events in the cell cycle.

Table 1. The position of nuclear division and cell plate formation in the cell cycle at different stages of exponential growth.

Cell concentration x 10^6	Nuclear division	Cell plate
1.35	0.78	0.86
1.73	0.75	0.85
2.4	0.70	0.87
4.5	0.74	0.86
4.5	0.73	0.86
5.15	0.75	0.86
7.2	0.75	0.85
8.75	0.80	0.86
Mean	0.75	0.86

Discussion

At first sight the observation that the mean DNA content of stationary phase cells is 53% of that of log. phase cells seems significant. It suggests that the cells are in G1 during stationary phase and that during log. phase DNA is synthesised quickly during the first part of the cycle. During exponential growth the culture will be composed of cells at all stages of the cell cycle. The mean DNA content of cells during this phase of growth will reflect the number of cells in G1, S and G2. If, for the sake of simplicity, the S phase is assumed to be an instantaneous event at the midpoint of the phase, and there is evidence presented in the following sections to suggest that it is very short in this organism, then the mean DNA content of exponential cells is determined by the proportion of G1 and G2 cells present in the samples. If we let a equal the proportion of G1 cells in the culture then (1 - a) will be the proportion of G2 cells, and if the stationary phase amount of DNA per cell (0.0199 μμg.) is taken to be the G1 amount, then the G2 amount will be 0.0398 μμg. DNA. The sum of the products of the amount of DNA and the proportion of cells in each phase should equal the product of the proportion of cells in the whole culture, 1, and the mean amount of DNA per cell for the whole culture. The following simple relation can therefore be written:

$$0.0199a + 0.0398\ (1 - a) = 0.0376 \times 1 \quad \text{equation (2)}$$

Solving this equation results in a = 0.111. That is to

say that 11% of exponentially growing cells would exist in G1 phase if the amount of DNA per cell during that phase is 0.0199 μμg. It can be calculated, if this proportion is substituted in equation (1) (derived in appendix 4), that the midpoint of the S phase is at 0.075 of the way through the cell cycle.

It is, however, extremely difficult to explain the results of the lag phase, or the low mean DNA content of spores, on the basis of this interpretation. There is another 1:2 ratio between the DNA contents of cells at different stages of the growth cycle. The mean value for spores is 50% of the mean value for cells in the plateau stage after inoculation. There is more justification for assuming the spore value to represent the DNA content of an unreplicated nucleus, and on this basis the single, haploid amount of DNA in S. pombe would be 0.0146 μμg. DNA/cell.

The terms haploid, diploid and polyploid have been used to describe different phenomena by different people. For example, diploid has been used to describe a nucleus that contains two sets of haploid chromosomes, each set being genetically different. Here the term diploid implies something more than just twice the haploid DNA content, rather that there are two sets of chromosomes which are genetically different from each other. On the other hand, diploid can refer to a replicated haploid nucleus, that is, one that contains two sets of

chromosomes that are identical. In this thesis the terms haploid or 1c will refer to the unreplicated nucleus and diploid or 2c to the replicated nucleus, in no way inferring the genetic implications of these terms.

If 0.0146 μμg. DNA/cell is the 1c amount, then the cells during the lag phase show a mean DNA content equal to the expected 2c value. After 3½ hours in the lag phase the DNA content of cells rises to a value approaching that of tetraploid cells. DNA synthesis must take place before cell division, because the rise in DNA content of the culture clearly precedes the increase in cell numbers, giving rise to the high DNA value per cell at this stage of the lag phase. As cell division proceeds the mean DNA value per cell drops towards that observed during normal logarithmic growth. This means that the log. phase population of S. pombe is composed of a mixture of 2c and 4c cells, DNA synthesis taking place towards the end of the cell cycle. It is important, though, to place it relative to nuclear division. If DNA synthesis is before nuclear division the nucleus must be 4c before it divides. This would be a rather unusual, though not impossible, situation. If synthesis was immediately after nuclear division the nucleus would be 2c for the majority of the cycle and it would not be necessary to invoke higher levels of ploidy to explain the results. It is rather difficult to answer this question using this system because of the

inaccuracies involved in the timing of synthetic events. There are, however, two observations which can be used to give some indication of the order of events, though they give conflicting results.

Firstly one can calculate the proportion of 2c and 4c cells in the log. phase culture, using equation 2 described earlier, though using different values for 1c, 2c and 4c, namely, 0.0146, 0.0292 and 0.0584 μμg. DNA/cell respectively. Thus:

$$0.0292a + 0.0584\ (1 - a) = 0.0376 \times 1$$

where a is the proportion of 2c cells and (1 - a) is the proportion of 4c cells. Solving this equation it is found that 71% of log. phase cells are diploid. Substituting this value for a in equation 1,

$$\frac{d}{T} = \frac{\log_e\ (1 - \frac{a}{2})}{\log_e\ 0.5}\ ,$$

it is found that the changeover from diploid to tetraploid, or the mid point of the S phase, occurs at 0.63 of the way through the cell cycle. This compares with 0.75 for nuclear division using the same method of positioning (see table 1), and as such suggests that nuclear fission follows shortly after DNA synthesis.

Some doubt as to the accuracy of this method is suggested by a study of nuclear division in Schizosaccharomyces versatilis (Robinow and Bakerspiegel, 1965). From their photographs it

appears as though nuclear division occurs half-way through the cell cycle. However, during and immediately after nuclear separation the two nuclei are not as easily visible as later on in the cycle. In the Giemsa stained slides of S. pombe there were usually cells in which it was not possible to see any nuclei. It may be that such cells represented those in which nuclear migration was taking place, and that when two nuclei became visible some time had elapsed since they had become separate entities. If this were the case in S. pombe then the estimate of nuclear division made on the basis of Giemsa stained cells would tend to position it later in the cycle than was really the case. Other errors will occur in the estimate of the times of the S phase and nuclear division. Accurate assessments depend on the assumption that the culture consists of a homogeneous population of cells dividing in complete asynchrony. That such a condition is not strictly the case is suggested by the work of Faed (1959) who shows, for example, there are variations in the cell plate index during log. phase. It is also probable that there will be individual variations between the cells with respect to the lengths of the different cell cycle phases. The ability of this method to give an accurate sequence of events in the cell cycle is doubtful, though it is useful in showing that nuclear division and DNA synthesis occur at similar positions towards the end of the cell cycle.

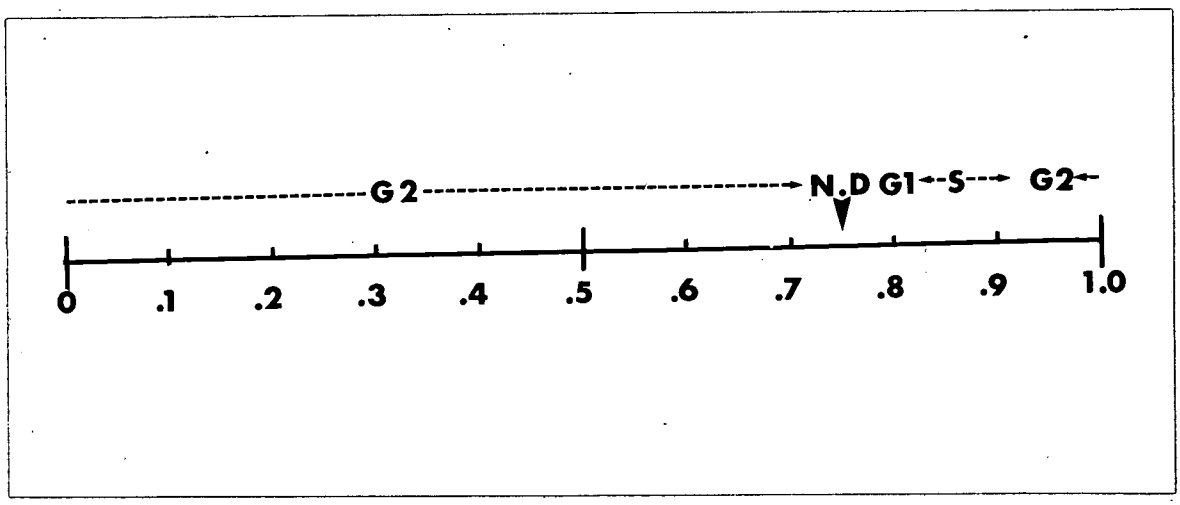
The second piece of information relevant to this question is the position of the increase in the mean DNA content of cells at the end of lag phase. It can be seen in figure 7 that the curve of DNA content per cell almost exactly parallels the curve of cell plate indices, and that, if anything, the peak in the DNA curve is slightly later than that of cell plates. Nuclear division must be before the formation of the cell plate, and therefore this would suggest that DNA synthesis occurs shortly after nuclear division. This observation carries more weight than the former, because here one is directly relating the position of the rise in DNA/cell to a cell cycle marker whose position is known. It is open to the objection that the lag phase is not a period of balanced growth, and as such the cell cycle might be distorted. There is little doubt that nuclear division must take place before the cells become physiologically separated, and thus in the lag phase DNA synthesis clearly occurs after nuclear division. That this is also the case in log. phase is supported by the results from synchronous cultures, which are described in the next chapter.

The results from the experiments on the various growth phases have yielded several pieces of information relevant to the description of the DNA synthesis cycle in S. pombe. They can be used to propose a scheme for the pattern of DNA synthesis and the one which best fits the facts and is most consistent

with the results in the following sections is as follows. The cell undergoes fission at 0.0 of the cell cycle, at 0.75 the nucleus divides, this being followed immediately by the synthesis of DNA which is completed before fission and possibly at the time that the cell plate is being laid down. At the time of cell fission a cell contains two diploid nuclei, giving rise to two daughter cells each with a replicated nucleus. There is therefore a long G2 period, a rapid S phase and an extremely short G1 phase, if indeed there is one at all. The implication of this is that at no stage during exponential growth do cells exist as a single unit in the G1 phase. This is shown diagrammatically in figure 9. The length of the S phase has been arbitrarily set at 0.1 of the cell cycle which is equivalent to about 15 minutes in absolute length. It is not intended that this is an accurate assessment of the length, though the results from the lag phase cultures and those from synchronous cultures suggest that it is extremely short and of this order of duration.

The only other report (Sando, 1963) of the DNA synthesis cycle in S. pombe agrees in only one respect to the results presented here. This is in the timing of the S period, which, Sando states, is at the time of nuclear division, but his values for the amount of DNA per cell, between 0.05 and 0.07 µµg. DNA/cell, are in excess of those found in this study. These differences could be due to the fact that Sando used a

Figure 9. Cell cycle map of S. pombe showing the proposed positions and relative lengths of the G1, S, G2 and M phases.


G 2
N.D
G1
S
G2
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0

medium which gave much higher yields of cells, and all his experiments were carried out at cell concentrations, which, in EMM 1, would be early to late stationary phase. Also, his synchronous cultures were in unbalanced growth, because none of the cellular components that he measured doubled over one cycle, nor did the waves of division bear any relation to the doublings of cell concentration.

The question now arises as to what happens when the cells enter stationary phase. Stationary phase cultures cannot consist of a homogeneous population of G1 cells, because the mean DNA content of stationary phase cells is significantly different from that of the spores. A high proportion of mitochondrial DNA could account for the difference, but that this is not the case will be shown in a later chapter. The stationary phase culture could be a collection of cells that had all synthesised part of their DNA before stopping growth. Such a phenomenon can be observed with the use of inhibitors, but has not been observed under normal growth conditions (Cummins and Rusch, 1966; Prescott and Stone, 1967). Alternatively, it could represent a mixed population of G1 and G2 cells. In this case some cells would have synthesised their DNA before they divided and stopped growing, whereas others would have divided without the normal prior synthesis of DNA, coming to rest in G1. If the latter were the case a rather unusual situation would have arisen. The order of three

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events that occur after nuclear division in the cell cycle would have been altered. Whereas normally the sequence is nuclear division, DNA synthesis, cell plate formation and cell fission, it would now become nuclear division, cell plate, fission and finally DNA synthesis. This is perhaps not strictly the case, because it could be argued that stationary phase cells are not growing, and that, rather than moving the time of DNA synthesis relative to the other events, it has just been omitted. If, however, the first hour of the lag phase is considered one observes a period of DNA synthesis sufficient to restore a mean DNA content per cell equal to the G2 value. If the stationary cells were a population of cells half-way through S then this initial synthesis would represent the completion of the S period. Alternatively it might represent the synthesis of DNA by unusual G1 cells, thereby restoring the normal diploid DNA value characteristic of log. phase cells. It is justifiable to consider the entry into stationary phase and the subsequent growth after dilution with fresh medium as part of a continuous, though distorted, cycle, because within a relatively short length of time the <u>status quo</u> of the log. phase cell is re-established throughout the culture.

Whichever of these two explanations is correct, the environmental conditions present in the medium as the cells enter stationary phase produce interesting alterations in the normal pattern of growth, which are completely reversible.

There is no way of distinguishing between them on the basis of the results so far, though the idea of an extended G1 is a more probable explanation in view of the fact that in other organisms it is the G1 phase which is most sensitive to changes in the cellular environment (e.g. Defendi and Manson, 1963; Richards *et al.*, 1956).

Chapter 3

Synchronous Cultures

Methods

The Synchronisation Procedure

The method used to produce synchronous cultures of S. pombe is that described by Mitchison and Vincent (1965), and is based on the differential behaviour of cells at different stages of the cell cycle while they are being centrifuged through a sucrose gradient. Depending on the size of synchronous culture required, 10 or 20 litres of culture were grown up in EMM 1 and harvested at a cell concentration of 3×10^6 cells/ml. by centrifugation. Cells were harvested in 250 ml. centrifuge bottles in an M.S.E. Major centrifuge. When large volumes were collected in this way the entire procedure took a long time, with the result that a proportion of the cells remained in a concentrated cell pellet for longer than was desirable. This had an effect similar to that when cells begin to enter stationary phase, and as such it had a slight effect on the synchronous cultures. At a later date the collection of cells was by continuous centrifugation which enabled the whole process to be quickened up considerably, and eliminated the slight distortions that occur at the beginning of the synchronous growth.

After harvesting, the cells were suspended in sufficient

medium to allow 5 ml. of the cell suspension to be placed on the top of each sucrose gradient. In practice one sucrose gradient was used for each 5 litres of starting culture. Linear sucrose gradients were set up in straight-sided 80 ml. centrifuge tubes by means of a gradient machine. The gradients were 70 ml. in volume and ran from 40% sucrose in EMM 1 at the bottom of the gradient to 10% sucrose in EMM 1 at the top. The concentrated cell suspension was carefully layered on the top of each gradient, and the tubes were spun for 7 minutes at 1500 r.p.m. in an M.S.E. Major centrifuge. The cells moved down the gradient in such a way that there was a distinct upper layer of small cells. This layer was removed from the gradient by means of a syringe with a long needle, which had a right angle bend at the tip. The end of the needle was placed at the lower limit of the layer of small cells, and liquid sucked into the syringe until the layer of small cells was transferred to the syringe. These cells were immediately inoculated into fresh EMM 1, and the cell concentration adjusted to about 3×10^6 cells/ml. The time that the cells were inoculated was taken as the start of synchronous growth.

In the control experiments the same procedure was followed up to the point that the layer of small cells was removed from the gradient. Instead, the gradients were shaken up so that a random distribution of cells was established throughout the 'gradient'. A sample of this was taken and inoculated into

fresh medium at a starting concentration of about 3×10^6 cells/ml.

The growth of the culture was followed in all cases by measurements of cell concentration, and in some cases by the estimation of cell plate indices. Samples for DNA assay were removed at regular intervals, harvested by centrifugation, and stored in the deep freeze until the assay procedure could be started on all the samples. In most experiments there was only sufficient culture to allow the estimation of the DNA content to be carried out over the space of one cell cycle. Most of the experiments were concerned with the first division after inoculation, but one was of the second division and another covered two divisions.

The Fluorometric Assay of DNA

The method which was used is a modification of that originally described by Kissane and Robbins (1958). It allows the estimation of between 0.1 and 10 µg. DNA and as such is about ten times as sensitive as the diphenylamine reaction. The original method has been altered considerably, so the method finally adopted will be described in detail.

Samples containing approximately $20 - 30 \times 10^6$ cells were removed from the culture, the cells were harvested, washed twice in distilled water, extracted with cold 5% trichloro-acetic acid (TCA) for 30 minutes, and washed twice in cold

5% TCA. The cells were then lipid extracted by suspending the cell pellet in ethanol, followed twice by ethanol-chloroform (3:1), ethanol-ether (3:1), and finally ether alone, after which the cell pellet was evaporated to dryness. The extraction of the lipids is important since many of these substances are strongly fluorescent and would interfere with the final assay. In order to make the method more specific for DNA it was decided to use DNase to 'extract' the DNA. As has been mentioned earlier DNase will not enter cells of S. pombe after cold acid or lipid extraction. The cells were therefore hydrolysed in 1 N KOH at 32°C for 6 hours. Acid insoluble material was then precipitated by the addition of 1 N TCA to make a final solution of 0.2 N TCA. The acid insoluble precipitate was collected by centrifugation at 15,000 g for 10 minutes. The precipitate was then treated in either of two ways. It was either washed in phosphate buffer (pH = 7.5) until the washings were neutral, or it was washed three times in 70% ethanol and dried. The former has the disadvantage that, for some reason, there is a greater risk of losing all the DNA, but when it works it gives much less scatter in the points. The ethanol washing tends to give consistent recovery of DNA but the estimates within one experiment show more scatter.

The precipitate is then resuspended in 0.05 ml. DNase solution (100 µg./ml. DNase in phosphate buffer, pH = 7.5, + 0.003 M $MgSO_4$) for three hours at 37°C. The suspension was

centrifuged at 15,000 x g for 10 minutes and the supernatant removed. The precipitate was washed once in buffer and re-centrifuged, the washing being added to the supernatant. The resulting solution was evaporated to dryness in a vacuum desiccator, the residue being taken up in as small a volume as possible of distilled water and again evaporated to dryness. The residue was stored in the deep freeze until the fluorometric reagent was prepared.

0.3 g. 3,5-diaminobenzoic acid (Sigma) was dissolved in 1 ml. 4 N HCl. This yielded a dark brown solution which was decolourised by five extractions with 0.01 g. activated charcoal. 0.05 ml. of the 3,5-diaminobenzoic acid hydrochloride solution was added to the residue and heated at 60°C for 30 minutes with the tubes tightly stoppered. The tubes were immersed in the water bath so that only the bottom tips of the tubes would be at 60°C; this allowed the rest of the tube to act as a condenser, helping to avoid complete evaporation of the small quantity of liquid in the tubes. 1.5 ml. 0.5 N PCA was added after cooling the tubes, which were then centrifuged at 15,000 g for 10 minutes to remove small amounts of dust and precipitate. The supernatant was removed and read at excitation wavelengths (primary) of between 405 and 410 mμ and an emission wavelength (secondary) of 510 mμ in an Aminco-Bowman double-sided spectrofluorimeter or a Locarte single-sided fluorimeter. In the latter an Ilford filter IL601 was used in

the primary beam and an IL624 in the secondary beam.

3,5-diaminobenzoic acid hydrochloride has two fluorophores, one that emits at 400 mμ and another that emits at 510 mμ, which is enhanced by reaction with purine deoxyribonucleotides (see figure 10). DNase also tends to react to produce fluorescence, but this is due to enhancement of the 410 mμ fluorophore. By the use of correct filters, or wavelength selection, it is possible to eliminate any interference from this source. However, some sources of DNase were found to produce strong fluorescence of the 510 mμ fluorophore and were thus unsuitable for the extraction procedure.

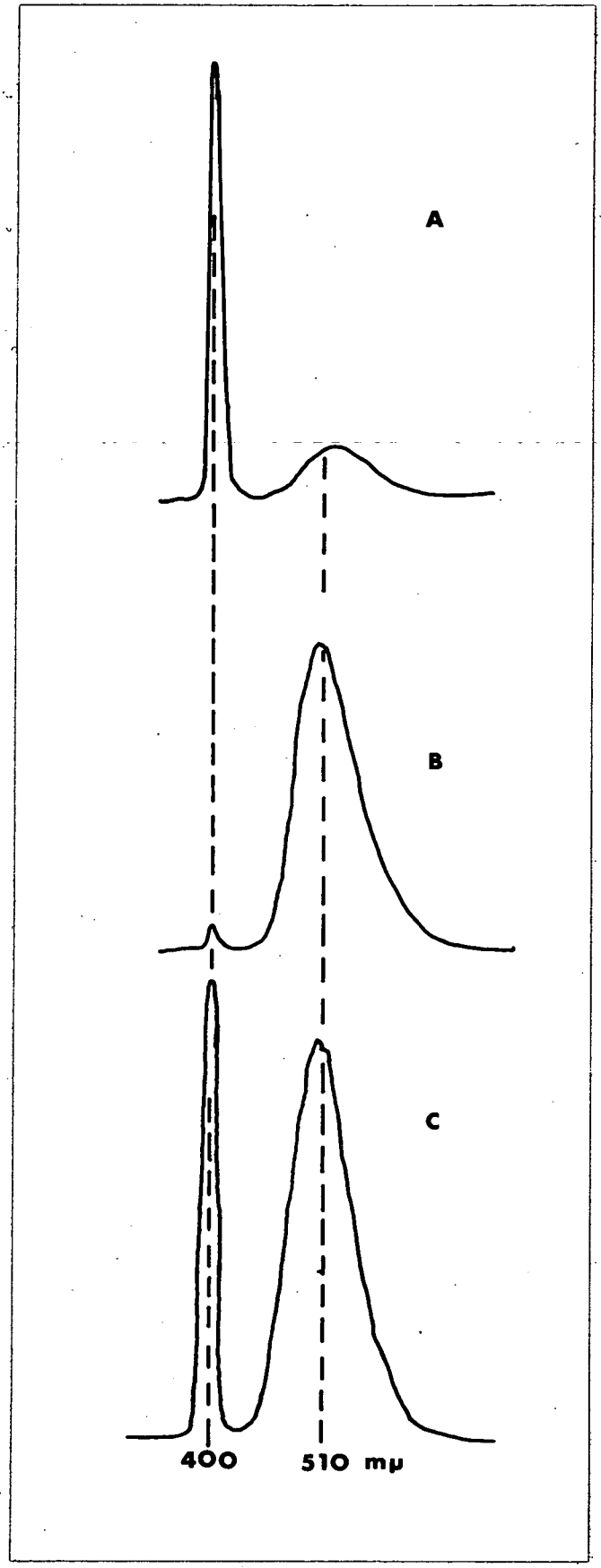
Experimental assays were always accompanied by a distilled water, phosphate buffer, and DNase blank, and a set of deoxyadenosine standards, all of which were evaporated to dryness before the reaction. As with the diphenylamine reaction, this method only estimates purine deoxyribonucleotides. Deoxyadenosine yields slightly more fluorescence than would be expected on the basis of the composition of DNA, and as such it was necessary to first calibrate the deoxyadenosine against DNase digested DNA.

Figure 10. The emission spectra of fluorescence produced by 3,5-diaminobenzoic acid hydrochloride when reacted with the residue of:

A. DNase in phosphate buffer (Scale expansion x1).

B. Standard deoxyadenosine solution (Scale expansion x1).

C. Distilled water (Scale expansion x10).


A
B
C
400
510 mμ

Results and Discussion

It is not possible to plot the combined results from several experiments on one graph, due to the variability in cell concentration, time of division, etc., between different cultures. Figure 11 shows the results of one experiment that was taken over two divisions. Several points emerge from this experiment. Firstly, the synthesis of DNA is periodic, as is shown by the curve of the amount of DNA/ml. of culture. Secondly, the period of DNA synthesis is at about the time of cell division. A close examination shows that the mid-point of the rise in DNA/ml. of culture just succeeds the peak in the cell plate index. Thirdly, the rise in DNA/ml. of culture is very sharp, and certainly as sharp as the increase in cell numbers. Fourthly, for the majority of the cell cycle, the mean DNA content of the cell is around 0.0304 μμg./cell and that this is very close to the suggested diploid value (0.0292 μμg. DNA/cell) for log. and lag phase cells. Lastly, when the DNA content/cell is plotted, one can observe increases at the time of division, which are very similar to the pattern found at the end of lag phase.

That these features are peculiar to cultures growing synchronously, and are not a product of the synchronising procedure, is shown by the control (figure 12). The growth in cell numbers shows a possible small initial lag, but after 30 minutes the culture is in exponential growth. The shape of

Figure 11. DNA per ml. (solid circles), DNA per cell (triangles) and cell concentration (open circles) in a synchronous culture of S. pombe.

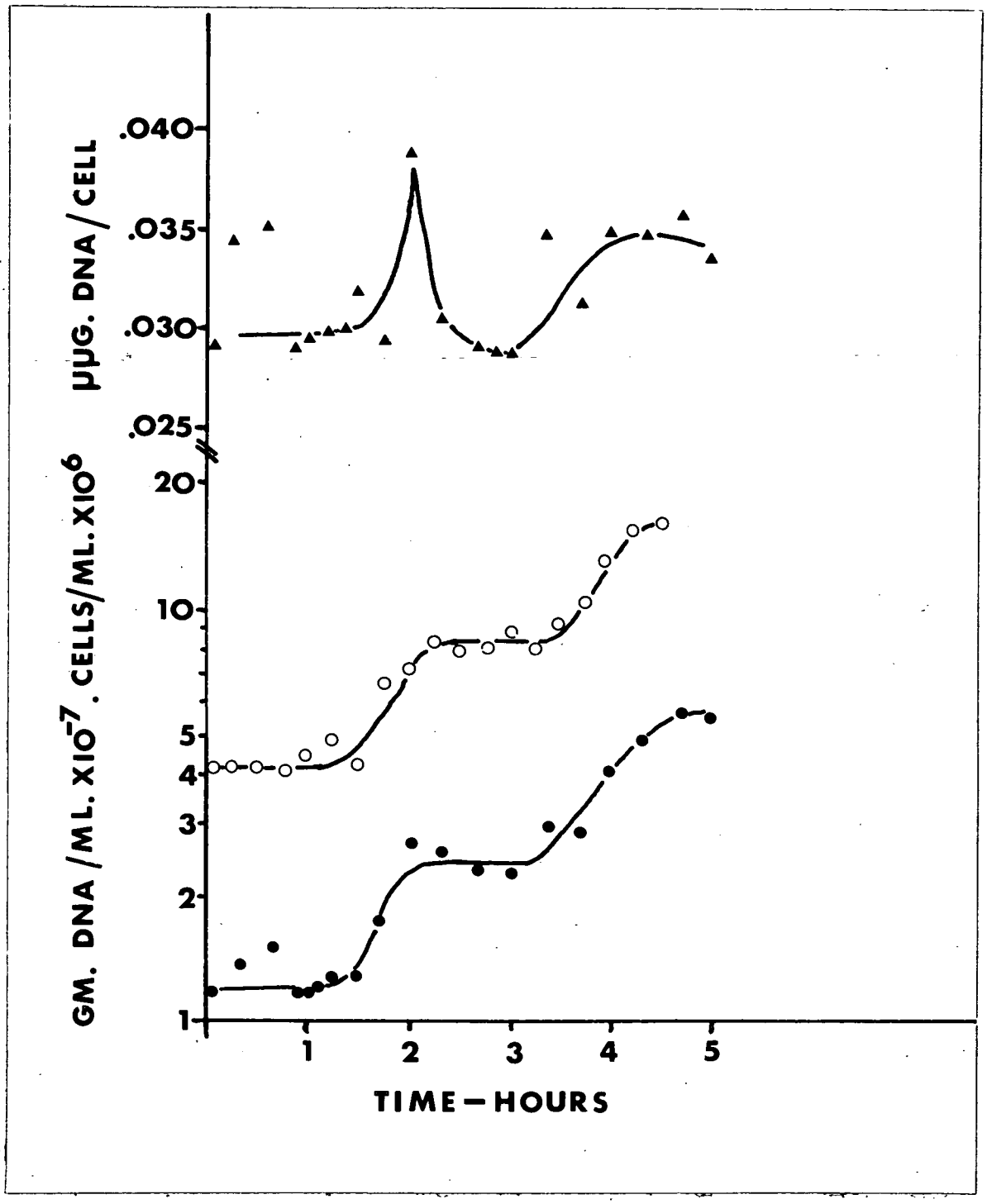
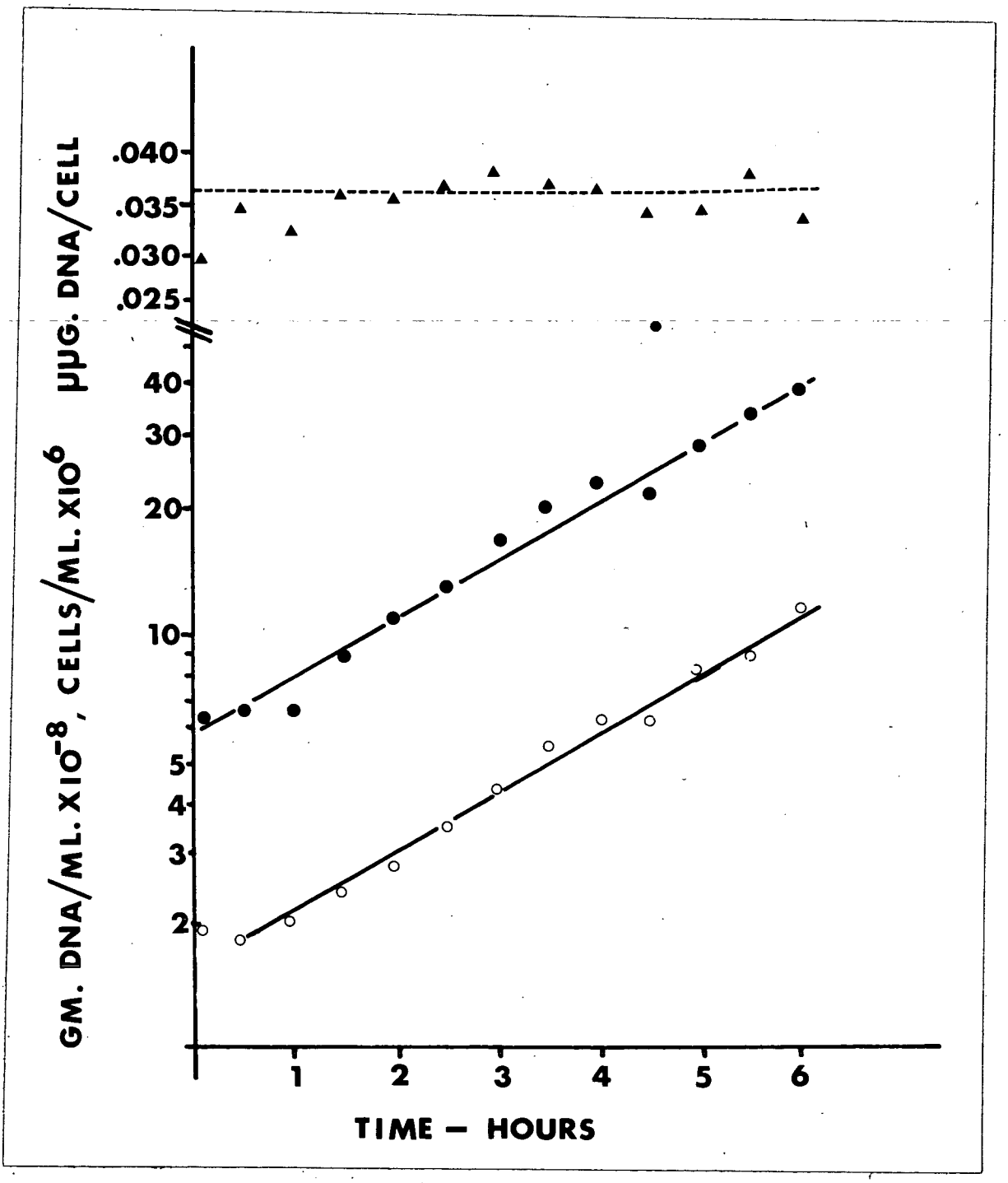

GM. DNA /ML. X10⁻⁷. CELLS/ML. X10⁶
μμG. DNA/CELL
.040
.035
.030
.025
20
10
5
4
3
2
1
1
2
3
4
5
TIME — HOURS

Figure 12. DNA per ml. (solid circles), DNA per cell (triangles) and cell concentration (open circles) in an asynchronous control culture of S. pombe.

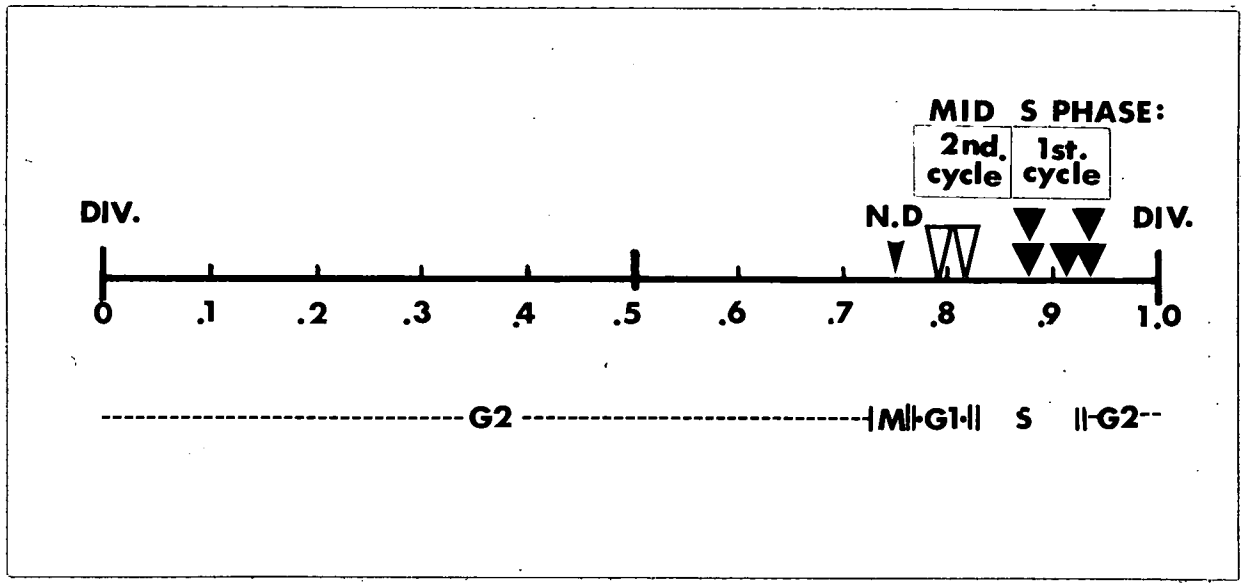

GM. DNA/ML. X10^{-8}, CELLS/ML. X10^{6} μμG. DNA/CELL
.040
.035
.030
.025
40
30
20
10
5
4
3
2
1
2
3
4
5
6
TIME — HOURS

the DNA/ml. curve is essentially similar. There is no periodicity in either cells/ml. or DNA/ml. and the mean quantity of DNA/cell remains fairly constant at 0.036 μμg., a value characteristic of random log. phase cells.

This result confirms the scheme proposed in chapter 2. Upon inoculation after the synchronisation procedure the cells are uniformly in G2. The cells grow up to the time of nuclear division, after which they are in G1 possessing two haploid nuclei, but still showing the mean diploid DNA value per cell. Shortly after nuclear division the S phase begins, and is half complete when the cell plate becomes visible. The S phase is completed before the cells undergo fission, which restores a uniform population of G2 cells. As was suggested in the previous chapter, there is a short (or absent) G1, short S and long G2 phase in the DNA synthesis cycle of this organism.

By considering the relationship between the mid-point of the rise of the DNA/ml. curve and the mid-point of the increase in cell numbers, it is possible to place, in the cell cycle, the point at which half the DNA is synthesised. Figure 13 shows the combined results of six experiments analysed in this way. One should not place too much emphasis on the exact positions shown on the map because they are derived by a method which is inevitably inaccurate. In order to arrive at the position it is necessary to draw a sigmoid curve through points which show a fair amount of scatter. It is not possible to

Figure 13. A cell cycle map to show the positions of the mid-point of the S phase in the cell cycle of S. pombe in different synchronous cultures.


MID S PHASE:
2nd. cycle
1st. cycle
DIV.
N.D
DIV.
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
G2
M
G1
S
G2

estimate precisely where the curve begins to rise or level off, and as such the slope of the curve and thus the mid-point of the rise will depend on the subjective assessment of the shape of the curve. Nevertheless all the estimates lie between nuclear division and cell division.

The estimates obtained from the second synchronous division are both earlier than those from the first division. This could be explained in terms of the stationary phase effect in random cultures. The collection procedure was long, and a proportion of the cells were maintained at a high cell density, which might have the effect of creating the limiting conditions necessary to induce the entry of cells into stationary phase. Table 2 shows that this is the case. DNA estimations were made at intervals during the harvesting procedure and it can be seen that the mean DNA content/cell drops as collection continues. This means that the half-time of DNA synthesis has been pushed back in the cycle so that DNA synthesis approaches fission. One might, therefore, expect to see slight disturbances in the timing of DNA synthesis in the first division cycle. The earlier position in the second cycle reflects a return to the correct position in the cell cycle. Another factor which could contribute to the different positions is the loss of synchrony as growth of the culture proceeds. It is therefore harder to assess the relative positions of the rises in number of cells or amount of DNA per sample.

Table 2. Decrease in the DNA/cell during the collection of cells for synchronisation.

Number of centrifugations	Mean DNA/cell (µµg./cell)
0	0.042
1	0.035
2	0.037
3	0.033
4	0.034
5	0.026

Chapter 4

Mitochondrial DNA

One of the assumptions, which is implicit in the reasoning so far, is that the measurement of the mean DNA content of cells is in effect an estimate of the nuclear DNA. Recently a considerable amount of evidence has been produced to show that the nucleus is not the sole DNA containing cellular organelle. Amongst the organelles that contain DNA, the mitochondrion is the most relevant to this thesis.

Nass *et al.* (1965) showed that mitochondria from a wide variety of organisms contain DNA-like fibres. The presence of DNA as such in yeast mitochondria has been shown by analytical ultracentrifugation studies on extracted DNA from whole cells and mitochondria (Tewari *et al.*, 1965; Corneo *et al.*, 1966; Moustacchi and Williamson, 1966). From the point of view of this work, the results of the latter authors has a particular relevance, because in that study the proportion of mitochondrial DNA varied according to the phase of growth of the culture. Moustacchi and Williamson showed that the proportion fluctuated from about 3% of the total during exponential growth to 20% of the total during stationary phase. This increase in mitochondrial DNA corresponds to the observed increase in the number of mitochondria over the same part of the growth cycle (Yotsuyanagi, 1962). These results were obtained on *S. cerevisiae*, but if

similar variations and proportions of cytoplasmic DNA were shown to occur in S. pombe a reassessment of the data presented to date would be required.

The purpose of the data presented in this section was to get some idea of the contribution that mitochondrial DNA was making to the estimates of total DNA in S. pombe. The results are inconclusive, but they do suggest that the mitochondrial DNA fraction in this yeast is rather smaller than in the budding yeast, though similar fluctuations occur as a culture passes through the different growth phases.

Methods, Results and Discussion

Before an analysis of the proportions of nuclear and cytoplasmic DNA could be made it was first necessary to find an effective method of disrupting the cells and, having achieved this, a suitable method of extracting the DNA. In previous studies on yeast DNA, cells were broken either by protoplasting and subsequent lysis (e.g. Moustacchi and Williamson, 1966), or by the process of slow freezing and thawing in the presence of Sodium Lauryl Sulphate (SLS) (Tewari et al., 1965). Several attempts to protoplast S. pombe cells both with the use of snail juice enzyme (e.g. Eddy and Williamson, 1959) or with various modifications of the method failed. It was found possible to obtain satisfactory yields of protoplasts on a small scale, where the ratio of snail enzyme to cells was high, but it proved

uneconomical to use this on a scale necessary to extract DNA from this organism. Attempts to protoplast the cells by growing them in the presence of 2-deoxyglucose (Johnson, 1968b) also failed to produce satisfactory yields necessary for the extraction of DNA. Freezing and thawing in the presence of SLS failed to rupture the cells, even after several cycles of the freezing process.

As a result of the failure of the above, methods of mechanical rupture of the cells were tried: grinding of a frozen cell pellet in a mortar and pestle in the presence of glass beads and passage of a cell suspension through an Eaton press (described in appendix 2). The most effective of these was the Eaton press which achieved about 95% breakage of the cells after one passage. The grinding of cells in the mortar resulted in a variable, though workable, cell breakage, but the resultant isolation of DNA from these homogenates gave very low yields. Because of this, and the fact that the grinding process is long and tedious, the Eaton press was used to rupture the cells.

The procedure adopted for the breaking of cells was as follows. 10 ml. of cell suspension, at a concentration of 10^8 cells/ml., in 10^{-2} M Tris/HCl buffer (pH = 8.2) were pipetted into the press, which had been pre-cooled in a dry ice/ethanol freezing mixture. The piston was replaced on top of the cells and the whole press put into position on the

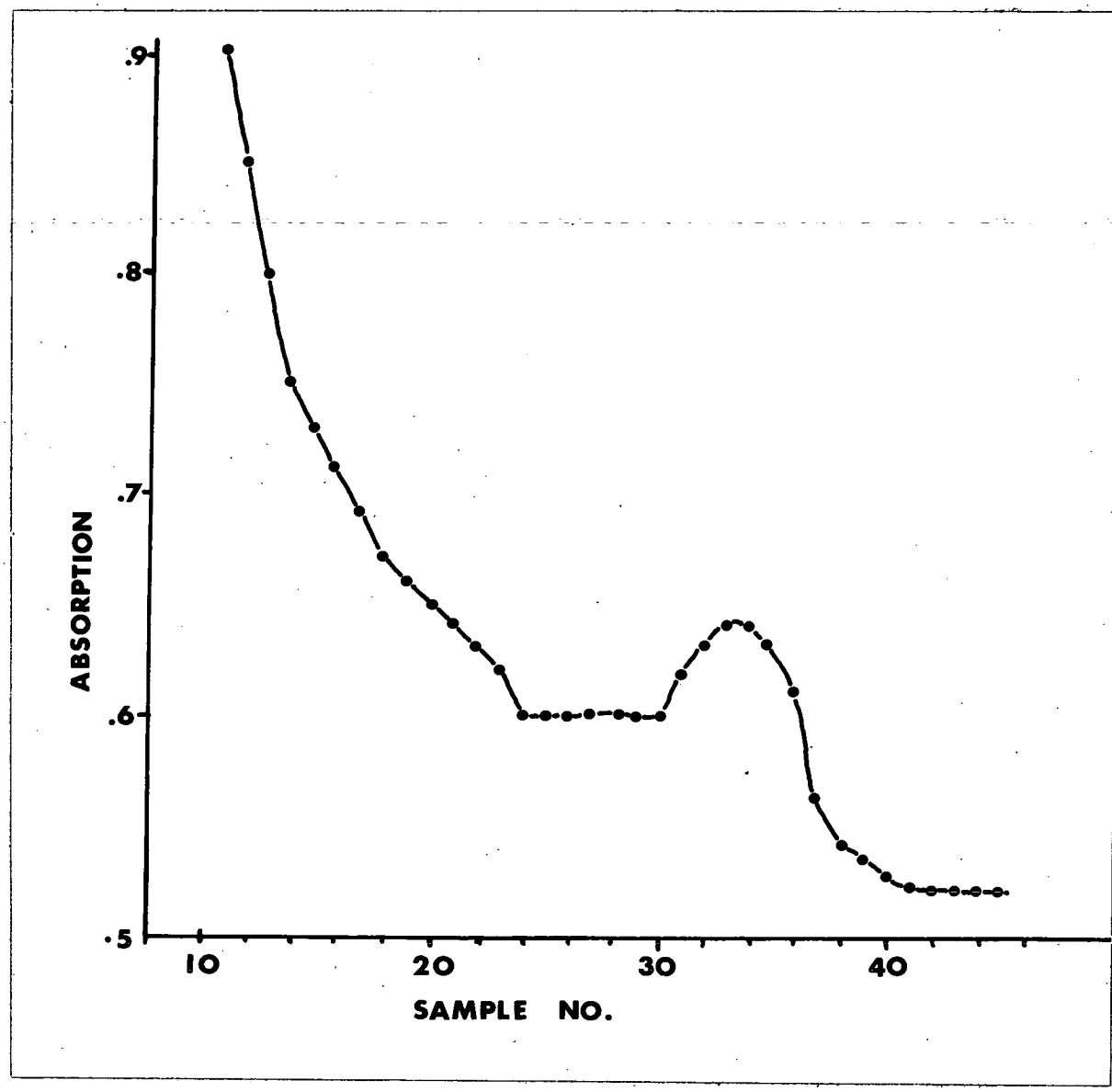
hydraulic press. After 5 minutes, to ensure that the cell suspension was fully frozen and at the same temperature as the press, pressure was applied to the piston. At a pressure of about 3-4,000 lbs./in.^{2} the cell suspension liquified and a small portion was expelled through the orifice into the collecting chamber. The loss of liquid resulted in a loss of pressure in the compression chamber, with the subsequent re-freezing of the cell suspension. The pressure was raised again until the process repeated itself. When all the suspension had been passed through in this way the press was dismantled and the collecting tube, containing the homogenate still frozen within it, was removed.

Several attempts were made to obtain satisfactory yields of high molecular weight DNA by the standard phenol or chloroform/octanol extraction procedures already widely used (Kirby, 1964). Although some DNA could be recovered by these methods the yields were disappointingly low, being of the order of 5%. The final method adopted was preparative isopycnic ultracentrifugation (Flamm et al., 1968), which is suited to the preparation of small quantities of DNA. It also had the great advantage that it separated a large proportion of the RNA from DNA due to the differences in their buoyant densities, thus making a ribonuclease digestion unnecessary.

The frozen cell homogenate was allowed to warm up to 0°C when it became manageably liquid. Caesium chloride was then

added, to make a final mean density of 1.720 gm./cm.^{3}, as soon as possible in order to stop the action of any nucleases that might have been present in the homogenate. The density of the CsCl solution was checked refractometrically, and the homogenate transferred to a 50 ml. polypropylene centrifuge tube, which was then topped up with liquid paraffin. The tubes were spun at 30,000 r.p.m. in an 8 x 50 rotor in an M.S.E. Superspeed 65 for 70 hours at 25°C, after which the rotor was decelerated without braking and carefully removed from the centrifuge. The tubes were removed from the rotor with care, their caps taken off and the gradients fractionated into 0.2 ml. samples by the simple device shown in appendix 3. The absorption of each sample was measured at 260 - 300 mμ in a Beckman DB spectrophotometer. An example of the change in absorption with the number of the fraction, and hence density of the solution, is shown in figure 14. It can be seen that there was a very high background of absorption due to unpelleted RNA and low molecular weight nucleic acid material. However, in the density range in which the DNA banded, sample numbers 24 - 40, the ratio of this material to DNA is of the order of 10:1, which is considerably lower than in the original homogenate. During the development of method the DNA content of the material banding, between fraction 24 and 40 in figure 14, was measured by the diphenylamine reaction. In all the experiments in which this was done the amount of DNA present in the pooled fractions could account for the peak in the absorption at this position of the gradient.

Figure 14. Change in absorption at 260mμ, minus the absorption at 300mμ, along the length of a caesium chloride gradient containing nucleic acid from S. pombe. Fractions are numbered such that the fir was collected from the bottom, most dense region, of the gradient.


.9
.8
.7
.6
.5
ABSORPTION
10
20
30
40
SAMPLE NO.

The DNA was further 'purified' by the pelleting process. The samples containing DNA were pooled and diluted with four times the volume of 10^{-2} M Tris/HCl buffer (pH = 8.2). The DNA was then pelleted by centrifugation at 100,000 x g for 18 hours, and the supernatant carefully poured off. Only high molecular weight material will be pelleted under these conditions, the mono-, di- and small polynucleotide material being left in the supernatant. The pellet was raised in 10^{-2} M Tris/HCl buffer (pH = 8.2) and left overnight at 0^{o}C to redissolve.

The resulting solution was spun for 30 minutes at 40,000 x g to pellet possible contaminants, e.g. glycogen, which have similar buoyant densities to DNA. CsCl was added to the supernatant to a mean density of 1.700 gm./cm.^{3} and the density checked refractometrically. This was spun on an M.S.E. analytical ultracentrifuge at 45,000 r.p.m. for 20 hours. Photographs of the resulting band pattern were taken and densitometer tracings made of them in a Joyce Loebel densitometer. The resulting tracings from three samples of DNA, extracted at different stages of the growth cycle, are shown in figure 15.

Tracing C in figure 15 is of DNA extracted from 24 hour stationary phase cells. It can be seen that there are two distinct peaks of ultraviolet absorbing material corresponding to two bands of DNA of differing buoyant densities. The major

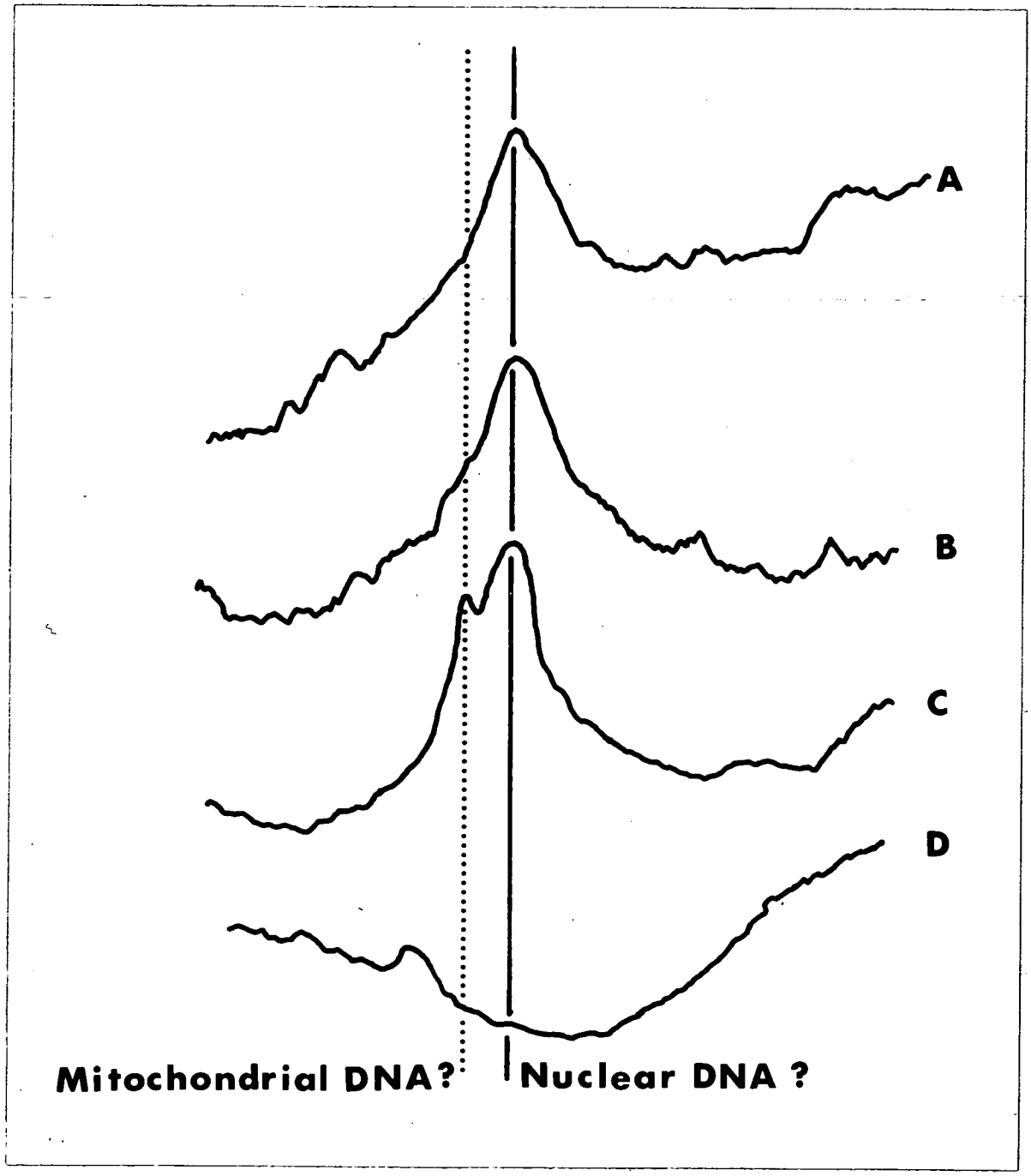
Figure 15. Densitometer tracings of the band patterns of DNA from S. pombe in the analytical ultracentrifuge.

A. DNA from early log. phase cells.

B. DNA from late log. phase cells.

C. DNA from stationary phase cells.

D. DNase digested DNA sample from stationary phase cells.


A
B
C
D
Mitochondrial DNA?
Nuclear DNA ?

peak, which has a higher buoyant density, would appear to correspond to the nuclear DNA of the more rigorous studies of Tewari et al. (1965), Corneo et al. (1966) and Moustacchi and Williamson (1966), while the minor lighter peak to the mitochondrial satellite band. Unfortunately no marker DNA was added to any of these samples, so it is not possible to determine the precise densities of these two components. Their relative positions on the gradient suggest that they differ in buoyant density by about 0.008 gm./cm.^{3}, which is less than that observed for S. cerevisiae nuclear and mitochondrial DNA. The difference may be slightly larger than this due to the fact that the satellite is on a rapidly increasing baseline, in this case the nuclear DNA peak, which would tend to move the satellite DNA peak closer to that of the nuclear DNA than it really is. Nevertheless this could not account for all the difference between S. cerevisiae and S. pombe. The smaller difference in the two buoyant densities of DNAs in S. pombe might not be all that surprising in view of the fact that the mitochondrial DNA of higher plant and animal cells has a density very similar to that of yeast nuclear DNA (e.g. Corneo et al., 1966).

That the peaks were DNA, and not some other cellular component of similar buoyant density, was shown by their absence in a deoxyribonuclease digested sample, tracing D in figure 15. Another indication that it was DNA that was

banding, rather than glycogen or related compounds, was the fact that the bands were invisible under Schlieren optics in the analytical centrifuge. Glycogen bands of sufficient concentration to show up in ultraviolet optics would have been easily visible with Schlieren optics.

Tracings A and B are of DNA extracted from cells at different stages of the growth cycle. The cells for A were at a cell concentration of 3×10^6 cell/ml., whereas for B the cell concentration was 1.8×10^7 cell/ml. Sample A represents log. phase cells and sample B late log./early stationary phase. From the tracings it can be seen that in A the minor DNA component is undetectable, whereas in B there are slight indications that it might be present. If this minor component is mitochondrial DNA these results suggest that, as in S. cerevisiae, the proportion of mitochondrial DNA changed as the cells entered stationary phase. The minor component was calculated to be approximately 8% of the total DNA in stationary phase. This compares with 20% for stationary phase in S. cerevisiae (Moustacchi and Williamson, 1966).

The validity of the method relies on the assumption that the proportion of nuclear and mitochondrial DNA in the extracts reflects the true proportions of the two within the cell. There is no way of telling whether this is in fact the case, because the extraction procedure could well be preferentially extracting one or other of the DNA types. Because of this not

too much emphasis can be placed on the absolute amounts of mitochondrial DNA in log. or stationary phase cells. However, because the same procedures were used throughout this study, it can be said that the amount of mitochondrial DNA increased by a factor of about eight as the cells entered stationary phase. The precise increase would depend on the amount of mitochondrial DNA present in log. phase cells, which is difficult to estimate from these results. Assuming about 1% mitochondrial DNA per log. phase cell, which, if the DNA content of a mitochondrion is about 1.6×10^{-10} µg. (Moustacchi and Williamson, 1966), is equivalent to about two mitochondria per cell, there is a relative increase of eight fold and is consistent with the results on S. cerevisiae. There are in fact very few mitochondria in log. phase cells grown under the same conditions that were used in these experiments (Schmitter, 1966), so a high proportion of mitochondrial DNA would not be expected in these cells.

In view of the results described above it was considered, subject to the assumptions implicit in the method, that the mitochondrial DNA fraction of S. pombe was not large enough to warrant a reappraisal of the scheme proposed to explain the results in the preceding two chapters.

Chapter 5

An Attempt to Prepare Autoradiographs of *Schizosaccharomyces pombe* for the Detection of DNA Synthesis

The results so far have been obtained by bulk biochemical assays on the hot PCA extracts of samples from randomly growing cultures or by fluorometric assay of the DNA content of synchronously dividing cells. The former suffers from the disadvantage that it measures the 'average' cell, which is difficult to interpret, and the second involves a long extraction procedure and an assay for small quantities of DNA, which tends to produce a lot of scatter in the points. The next part of the thesis is an investigation of possible changes to the DNA synthesis cycle, and if the alterations were small compared to the whole cycle it would be difficult to identify with the biochemical methods used to date. Before commencing with these experiments an attempt was made to prepare autoradiographs to investigate the time of DNA synthesis, in the hope that if it proved possible it might give a method which could be used in the detection of small changes to the DNA cycle.

The standard method for the detection of DNA synthesis by the incorporation of a radioactive label into the macromolecule is by supplying the cells with tritiated thymidine in the medium. In most organisms this labelled precursor is

specifically incorporated into DNA. In S. pombe this is not so, because it is also incorporated into RNA and a proportion of the labelled RNA is resistant to RNase treatment yielding an even distribution of grains in the autoradiograph over the cytoplasm (Mitchison, 1963a). During the course of the work for this thesis Williamson (1965) published a method for the autoradiographic analysis of DNA synthesis in the budding yeast, Saccharomyces cerevisiae. This was based on the non-specific incorporation of labelled adenine into all nucleic acids, followed by the specific removal of RNA. It was decided to try this method on S. pombe to see whether, using the same extraction procedure or slight modifications of it, it would prove possible to remove all the labelled RNA from cells. The majority of the work was done on whole samples of cell suspensions following the incorporation of 14C-adenine, because it was not only quicker, but gave more accurate assessment of where the label was being incorporated and retained. The experimental procedure was as follows.

20 ml. of log. phase culture (3×10^6 cells/ml.) were transferred to a tube containing 0.05 ml. 14C-adenine (500 µg./ml.), specific activity 31.3 mC/mM, to give a final specific activity of 0.29 µc/ml. The tube and cell suspension were incubated at 32°C for 20 minutes after which the tube was placed in crushed ice and the cells collected on a membrane filter, and washed several times with ice cold distilled water

whilst they were still on the filter. Following this the cells were resuspended in 5% PCA in the cold (4°C) for 30 minutes, and subsequently washed several times in distilled water. The cold PCA extract was retained for the estimation of radioactivity that it contained. The cells were then extracted with RNase for 2 hours at 37°C to remove the bulk of the label in the RNA. Five times crystallised bovine pancreatic RNase (Sigma) was used at a concentration of about 200 µg./ml. in tris/HCl buffer, pH = 7.6, after the enzyme solution had been placed in a boiling water bath for 10 minutes. The supernatant containing the RNase digestible material was retained and the cells washed several times in distilled water. This was followed by treatment of the cells for 30 minutes with 0.3% (w/v) formaldehyde in phosphate buffer, pH = 7.0, at room temperature. After several washes the cells were suspended in 1 N NaOH for 1 hour at 25°C and the supernatant again retained for estimation of labelled material. The alkaline digestion was followed by several washes with distilled water and a protease digestion with trypsin and chymotrypsin (Sigma), at concentrations of 250 µg./ml. in tris/HCl buffer, pH = 8.0, for 2 hours at 37°C. After more washings in water a final DNase digestion was carried out at a concentration of 100 µg./ml. in tris/HCl buffer, pH = 7.5, containing 0.003 M $MgSO_4$ at 37°C for 2 hours. As with the other treatments the supernatants remaining after extraction with proteases and DNase, and the residual cell fraction, were retained for the measurement of

the radioactivity they contained. Measurements of radioactivity were made by drying down aliquots of the supernatants on to planchets and counting in an 1DL Automatic Low Background Counter, Type 2106.

Table 3 shows the percentage of the total amount of radioactivity taken up by cells that each fraction during the extraction procedure contained. It can be seen that by far the largest fraction of incorporated 14C-adenine was in RNase sensitive RNA, but the important points are that a significant amount was incorporated into protein and also a portion, slightly less than that in DNA, remained in the cell residue at the end of the extraction procedure.

Similar experiments were performed with 3H-adenine, with the preparation of autoradiographs by the method of Cummins and Mitchison (1964). The only differences between the procedure described above and that used for the preparation of autoradiographs was that the final concentration of 3H-adenine in the medium was 10 μc./ml., and the extraction procedure was finished before the DNase step. Autoradiographs were exposed for varying lengths of time between 14 days and three months, but in all cases there was an even distribution of grains over the cytoplasm (see figure 16).

Variations were made in the procedure, by increasing the length of RNase extraction, varying the length of formaldehyde

Table 3. Distribution of 14C-adenine in different fractions during the procedure for removing labelled RNA.

Fraction	Counts/100 sec.	Percentage label in each fraction
Whole cells	6178	100
Cold PCA soluble	467	7.23
RNase sensitive	5815	89.97
NaOH "	82	1.27
Protease "	18	0.28
DNase "	45	0.70
Residue	36	0.55

Figure 16. Photographs of autoradiographs of S. pombe labelled with 3H-adenine and treated as described in the text for the removal of RNA and protein. These autoradiographs were exposed for 6 weeks.

10μ

fixation and increasing the length of NaOH extraction in an attempt to remove the residual radioactivity remaining after DNase digestion. Any method which achieved this in bulk extraction resulted in the destruction of the cell, and attempts to make autoradiographs using the same procedures were abortive because of the inability to identify cells.

Similar procedures were also used after the incorporation of labelled thymidine into cells of S. pombe, but essentially the same results were obtained. Because of the inability to remove the residual radioactivity and yet retain the identity of the cell it was decided to abandon further attempts to make autoradiographs and use the biochemical assay procedures described previously.

Chapter 6

Alterations to the DNA Synthesis Cycle - An Introduction

The results described in the previous chapters demonstrate the normal pattern of DNA synthesis in S. pombe, when it is grown on Edinburgh Minimal Medium 1 at 32°C. This can be related to the other known patterns of macromolecular synthesis and cell cycle events that have been observed under the same growth conditions. Such an analysis allows one to say that there are certain sequences of events through the cell cycle, but it does not permit one to say that there is any direct causal relationship between them: the only relationship that is observed is that each is related to the others by a position in time. In order to establish the degree of causality between events it is necessary to distort the cell cycle, and alter in some way the position of one or more of the events, and to observe the effect on the other events that one is interested in.

In studies of the control of macromolecular synthesis the behaviour of the genetic material is of fundamental importance. Ultimately all cellular synthetic systems are under genetic control, but the degree of directness of this control is, to some extent, an unknown quantity. One way of approaching this problem would be to alter the DNA synthetic cycle and see what

changes, if any, are brought about in other synthetic systems.

There are several ways of approaching the experimental alteration of the DNA synthesis cycle. Under conditions that are sub-optimal in some instances the various phases of the cycle appear to lengthen differentially. Amongst the environmental factors that can bring about such a change in the DNA synthesis cycle are temperature, pH, nutritional factors, especially alterations in the carbon source, and the use of inhibitors.

The effect of altering the growth rate of the cells, by changing the growth temperature, on the DNA synthesis cycle has been studied in several organisms. The experiments on mammalian cells in culture showed that all the phases of the cycle were temperature dependent, there being absolute increases in all of them if the temperature was raised or lowered from that which was optimal (Rao and Engelberg, 1966; Sisken et al., 1965; Watambe and Okada, 1967). That each was affected by temperature shifts is not surprising, because most synthetic processes show strong temperature dependence. What is significant is the fact that the G1 phase showed a greater increase in length than the other phases, when cells were cultured at lower temperatures. The results of Sherman et al. (1961), on the DNA synthesis cycle of mouse ear epidermis, can also be interpreted in terms of an extension to the G1 phase due to a lower environmental temperature. The same phenomenon of an

extended G1 phase, associated with increased generation times due to temperature changes, has also been shown in the cells of Tradescantia root tips (Wimber, 1966), and for lens epithelial cells of the bullfrog (Reddan and Rothstein, 1967).

The differential effect observed in higher plant and animal tissues has not always been observed in lower organisms. In bacteria alterations in the generation time by temperature alone had no effect on the amount of DNA per cell (Schaechter et al., 1958), and, though this was not a particularly sensitive method for showing small changes in the timing of DNA synthesis, it was sensitive enough to show the changes due to growth on different carbon sources. There is also evidence that temperature has an effect on the DNA synthesis cycle in Tetrahymena pyriformis, though the results are conflicting. MacKenzie et al. (1966) showed that the various phases were equally temperature dependent, whereas Cameron and Nachtwey (1967), who used the same strain of T. pyriformis grown under the same conditions, showed that the G1 was more temperature dependent than S, which remained fairly constant in absolute terms, and G2. Cleffman (1967), again using the same strain but grown on a more enriched growth medium, showed that all phases in this organism were equally temperature dependent with a Q_{10} equal to 2. The temperature sensitivities of G1, S and G2 are not clear when this organism is in balanced growth, but experiments using the temperature shift method for synchronisa-

tion showed that DNA synthesis can be dissociated from the division cycle (Zeuthen, 1963). In these experiments the change was not a stable one, but the relative lengths of G1, S and G2 were altered. It is therefore possible to alter the DNA synthesis cycle in T. pyriformis by temperature shifts alone.

In S. pombe Mitchison et al. (1963) have shown that patterns of growth can be changed by reducing the culture temperature from 32°C to 17°C. In their measurements on single cells grown at 17°C they showed that the rate of increase in dry mass fell to zero over the last portion of the cell cycle. Under optimal growth temperatures the majority of increase in dry mass takes place in G2 in this organism, the G1 phase being very short. It may be that the majority of increase in dry mass at 17°C is still occurring in G2, and the falling to zero of the rate of increase reflects the presence of a measurable G1 phase, a G1 which is more sensitive to the increase in the generation time than either S or G2. This is one indication that temperature changes could be affecting the DNA synthesis cycle in S. pombe.

Changes in the composition of the culture medium can also bring about differential changes in the DNA synthesis cycle. This might be inferred from the studies on similar cells at different developmental stages or different areas of the same tissue. Marchok (1965) studied DNA synthesis in relation to

the division cycle in developing chick leg muscle. Between the ninth and sixteenth days after hatching the cell generation time had increased from 11.5 hours to 16 hours, yet the S and G2 periods occupied the same length of time, indicating that G1 had been extended. Clowes (1965) showed that the large difference in the generation times of the cells in different areas of the root meristem of Zea mais was almost entirely due to differences in the relative lengths of the G1 phase. Similar results have been found in the mesenchyme cells of growing rat tibiae (Young, 1962). In this tissue the cells in different regions had different generation times, and, though there was an absolute increase in the length of the S and G2 phases with increased generation time, the majority of the change was accounted for by a differential lengthening of G1. More direct evidence of the effect of nutritional or pH changes comes from the work on tissue culture cells (Defendi and Manson, 1963; Sisken and Kinosita, 1961). Both these reports support the hypothesis that G1 is relatively more susceptible to changes in the environment than either S or G2.

Work on this aspect of micro-organisms has mainly centred around the bacteria, which has been discussed in chapter 1. To summarise this work: alterations in the carbon source of the medium, which result in slower growth rates, lower the mean amount of DNA per cell, which can be interpreted as a relative lengthening of the pre-initiation phase of the DNA synthesis

cycle. There is also evidence, though, that nutritional factors affect G1 differentially in Euglena and T. pyriformis. Wilson and Levedahl (1964) showed that if Euglena was grown on synthetic media containing different carbon sources, the mean DNA content of cells decreased as the generation time increased, indicating that there was a longer interval between division and DNA synthesis in the slower growing cells. For T. pyriformis previous reports of the DNA synthesis cycle indicate that less enriched media, which support longer generation times, result in the macronuclear S period being shifted from early interphase to the middle of interphase (Cameron and Stone, 1964; Stone and Cameron, 1964; Stone and Prescott, 1964). This has been substantiated by Cameron and Nachtwey (1967) who showed that at slower growth rates the S phase increased slightly in length and moved to a later position in interphase, showing that G1 was the most sensitive phase.

There are few ways of increasing the generation time of S. pombe by alterations in the composition of the medium. The generation time of S. pombe on defined medium is very similar to that on complex medium ('Oxoid' Malt Extract Broth), and the mean DNA content per log. phase cell is the same in both media, indicating very similar patterns of DNA synthesis. Unfortunately there are very few sources of carbon that will support growth of S. pombe, and most of those that do, support similar generation times. Thus, before the effect of nutritional changes could be

investigated, it would be necessary to find conditions that would support slower balanced growth in this organism.

The mixed stationary phase population in EMM 1 suggested another approach to the problem of altering the DNA synthesis cycle with changes in the composition of the medium. If this population is genuinely a mixture of G1 and G2 cells there must be some factor which is selectively lengthening G1 in a proportion of the cells. If this factor could be identified it might prove possible to grow continuous cultures under the same limiting conditions as those that cause the cells to enter stationary phase. Under such circumstances the culture would be in balanced growth, and yet the various phases of the DNA synthesis cycle might be altered in much the same way as when cells enter stationary phase in EMM 1.

Another way of altering the cell cycle is by the use of specific metabolic inhibitors. Essentially there are two ways of approaching this. An inhibitor, which is specific for DNA synthesis, can be used and then observations made on its effect on the other events in the cell cycle. Alternatively the effect of other macromolecular synthetic inhibitors on the DNA synthesis cycle can be studied. Examples of the latter are amino acid starvation of bacterial auxotrophs (Lark, K., 1966), the use of methionine analogues in E. coli (Lark, C., 1968b), the deprivation of essential amino acids in T. pyriformis (Stone and Prescott, 1964), the effect of actidione on Physarum

polycephalum (Cummins and Rusch, 1966), the effect of puromycin on mouse fibroblasts (Littlefield and Jacob, 1965) and the eventual inhibition of DNA synthesis by actinomycin D in Ehrlich Ascites cells (Baserga et al., 1965). There are many more examples, which are too numerous to go into detail here. However, more emphasis will be placed on the former approach, since this thesis is primarily concerned with the DNA synthesis cycle, and the ways in which it can be directly altered.

Several techniques and substances have been used in this particular field of cell cycle analysis. There are the alkylating agents of which Mitomycin C has been fairly widely used (Loveless, 1966; Waring, 1966). This antibiotic acts by forming cross links between complementary strands of the DNA molecule, thereby interfering with the replication process (Iyer and Szybalski, 1963; Lawley, 1966). It is principally specific to DNA synthesis, though eventually other synthetic systems within the cell begin to break down and there are reports of it affecting ribosomal RNA and protein (Waring, 1966). This inhibitor, and those with similar modes of action, might be said to have irreversible effects on cells in as much as the withdrawal of the inhibitor is not the sole factor in the recovery process. For cells to recover from the effects of these inhibitors it is necessary that they have the mechanism which excises the cross-linked DNA and replaces it with a new, correctly coded piece of DNA. It is possible that the

mechanisms involved are the same as those responsible for the repair of DNA damage following ultraviolet irradiation (Waring, 1966; Lawley, 1966). This process necessarily takes time and results in division delay which would complicate the basic effect on DNA synthesis.

Other inhibitors appear to be completely reversible in their effect on DNA synthesis. Nalidixic acid is one that has been shown to be reversible in E. coli, where it was specific for DNA synthesis, the synthesis of RNA, protein and lipid being unaffected (Gross et al., 1964; Kantor and Deering, 1968). In both these reports nalidixic acid resulted in the loss of some DNA from treated cells, which is clearly undesirable. The latter authors also investigated the effects of another inhibitor, hydroxyurea. It was shown to be specific for DNA synthesis as well reversible in its action. It did not cause degradation of DNA (Kantor and Deering, 1968), but it has been reported to be lethal to cells in the S phase (Sinclair, 1965). In this study it was shown that hydroxyurea inhibited the entry of cultured mammalian cells into the S phase, without stopping their progression through G1 and G2. This resulted in a build up of cells just prior to the S phase, but caused the death of those that were in S at the time of addition of hydroxyurea.

Another way of specifically inhibiting DNA synthesis has been the withdrawal of thymine from thymine auxotrophic

bacteria (reviewed by Donachie and Masters, 1968). In the absence of a thymine requiring strain the cells can be artificially starved of thymine by the addition of 5-fluoro-2-deoxyuridine (FUdR). The effect of this was first demonstrated by Cohen *et al.* (1958) and it has since been used as a specific inhibitor of DNA synthesis in several organisms. Furthermore its inhibitory effect can be reversed by the addition of excess thymidine to the medium. Sachsenmaier and Rusch (1964) showed that it inhibited DNA synthesis in *Physarum polycephalum*, and Till *et al.* (1963) used this effect to synchronise L strain mouse cells. FUdR was not lethal to cells in S phase, unlike hydroxyurea, and upon addition of thymidine to the medium the S phase cells started to grow from the point at which they were blocked. FUdR has also been shown to be effective in *Bacillus subtilis* (Donachie and Masters, 1965). Unfortunately this inhibitor seems to have only a limited effect on *S. pombe* (Herring, pers. commun.), and is at present being investigated by him.

2-phenyl ethanol (PE) is also reported to be a specific inhibitor of DNA synthesis by some workers. The initial report of the inhibitory action of PE on DNA synthesis was on *E. coli* (Berrah and Konetzka, 1962). This view has since been supported by work on *E. coli* (Lark, K., and Lark, C., 1966; Trieck, 1966), mouse L cells (Bruchovsky and Till, 1967) and Ehrlich IIB cells (Leach *et al.*, 1964). All these reports

supported the idea that, at the right concentrations, PE exerted a specific and reversible inhibition on the synthesis of DNA. The work on E. coli further suggested that it was the initiation of the S phase which was the sensitive part of the cell cycle, because rounds of replication, that were in progress at the time of addition, were completed (Lark, K., and Lark, C., 1966; Trieck, 1966).

There is another 'school' that suggests that the primary site of PE inhibition is RNA synthesis (Provost and Moses, 1966), and messenger RNA in particular (Rosencranz et al., 1965). Further support for the site of inhibition being mRNA comes from the work of Slepecky and Celkis (1964) who showed that the sporulation of Bacillus species was inhibited by PE after growth of the culture had ceased.

Despite this conflict it was decided to investigate the effect of this inhibitor on S. pombe, to see whether it would be possible to alter the DNA synthesis cycle. If the site of action is indeed DNA synthesis, with little effect on the other macromolecular synthetic systems, and if its action is completely reversible, then, by periodic additions and withdrawals of PE, it might prove possible to artificially move the S period to a different part of the cell cycle. As will be seen in the section on the effect of this inhibitor, S. pombe reacts to the presence of certain concentrations of PE in an unique way, and the periodic addition and withdrawal of PE did not prove necessary.

Chapter 7

The Phosphate Effect

Methods

RNA Estimations

The RNA content of samples was estimated on the hot 5% PCA extracts by measuring the absorption at 260mμ and subtracting from it the absorption at 300mμ in a Unicam SP 500 spectrophotometer. Cells of S. pombe contain approximately 100 to 150 times as much RNA as DNA so 99% or more of the absorption at 260mμ will represent the absorption due to RNA.

Phosphorus Determinations

30 ml. samples were removed from the culture and the cells harvested by centrifugation. The EMM supernatant was carefully decanted and retained, and the cells washed three times with distilled water. Following the washes 5 ml. of distilled water was added to the cell pellet and the resulting cell suspension slowly frozen in a deep freeze. This was followed by slow thawing of the cell suspension, centrifugation and removal and retention of the supernatant. The cell pellet was washed once with distilled water, the washing being added to the original 5 ml. supernatant. This fraction was considered to be the 'pool', consisting of, amongst other substances, the low molecular weight phosphorus containing compounds of the cell.

Both this fraction and the original sample of the medium were evaporated to dryness and the resulting residues were raised in 1 ml. distilled water. 0.1 ml. of this was added to 0.9 ml. 72% PCA (Analar) and 1.0 ml. of 72% PCA was added to the frozen/thawed cell pellet. All the tubes were 'capped' with glass marbles and heated carefully on a microburner until the contents were colourless.

To these samples were added 10 ml. distilled water, 1 ml. 5% (w/v) ammonium molybdate and 0.5 ml. reducing agent. The latter consisted of 0.2% (w/v) 1,2,4-aminonaphthol sulphonic acid, 12% (w/v) sodium metabisulphite and 2.4% (w/v) sodium sulphate in 100 ml. distilled water. As this solution had to be made up fresh each time the three components were mixed in the correct proportions and ground up in a mortar and pestle to yield an even mixture. When required, 14.6 gm. of this mixture were dissolved in 100 ml. of distilled water.

After the reagents had been added and well mixed, the tubes were left to stand for 10 minutes before the absorption at 830 mu was measured in a Unicam SP 500 spectrophotometer.

Standard solutions of sodium dihydrogen orthophosphate and distilled water blanks were taken through the ashing procedure before the amounts of phosphorus were estimated. Standard curves were constructed and the amount of phosphorus present in the different samples was found by interpolation. The results

in the following section are expressed in terms of phosphate rather than phosphorus.

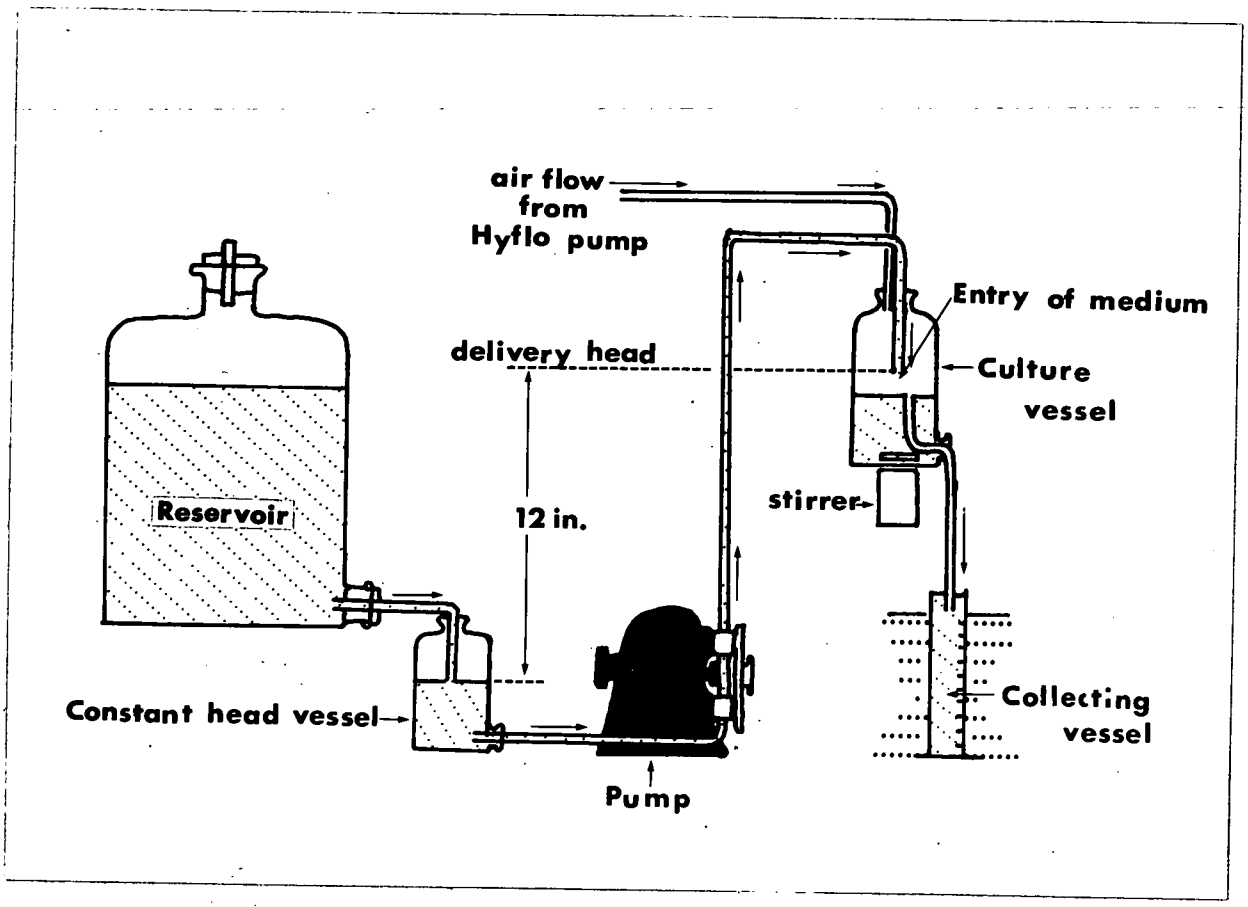
Viability Counts

The estimates of the number of viable cells were made by the method of Swann (1962) in his studies on the effect of ultraviolet light on single cells. The suspension of cells was shaken vigorously, to disperse the clumped cells as much as possible, and a small quantity, a bacteriological loopful, was spread evenly over a thin agar pad on a microscope slide. A coverslip was then placed on top and sealed in position with paraffin wax. A small capillary tube was also sealed into the wax in such a way that it allowed free exchange of gas between the cells and the outside. The cells were then observed under a microscope, which was contained in a constant temperature box, at 32°C. Photographs were taken every hour, for 24 hours, by means of a time-lapse device, giving a record of each cell in the microscopic field. In this way it was possible to monitor accurately the viability of about 200 cells per microscopic field. Although it suffered from the disadvantage that only a small number of cells could be monitored, it did avoid the major difficulty of plating techniques and colony counting, where it is hard to assess the number of clumps and pairs in the original cell suspension. With this photographic method cells were considered to be inviable if they had not divided within 24 hours.

Continuous-flow Culture Apparatus

Figure 17 shows diagrammatically the apparatus used to continuously culture S. pombe. It was a crude version of the standard type of apparatus used for this purpose (Malek and Sencl, 1966), and consisted essentially of four parts. There was a reservoir which supplied medium low in phosphate to the culture chamber by means of a constant rate flow pump, which could be adjusted to suit the cell generation time required. The culture chamber contained 500 ml. of culture medium, which was vigorously stirred with a magnetic stirrer, and maintained at a constant volume by means of an overflow device. This was essentially an open glass tube set to the height required. There was a tendency for the culture medium to build up around the opening of the tube due to surface tension. This was, to a large extent, eliminated by having a constant stream of sterile air passing over the culture and down the overflow tube. This also served to aerate the culture. Samples were collected as the effluent coming out of the culture in a vessel packed in ice in an insulated container, the whole apparatus being kept in a warm room at 32°C.

Variations in the growth rate and yield of the culture were obtained by alterations in the phosphate concentration in the medium or the flow rate of fresh medium into the culture. The culture was started with a small inoculum while the flow of medium through the culture chamber was in progress. At low

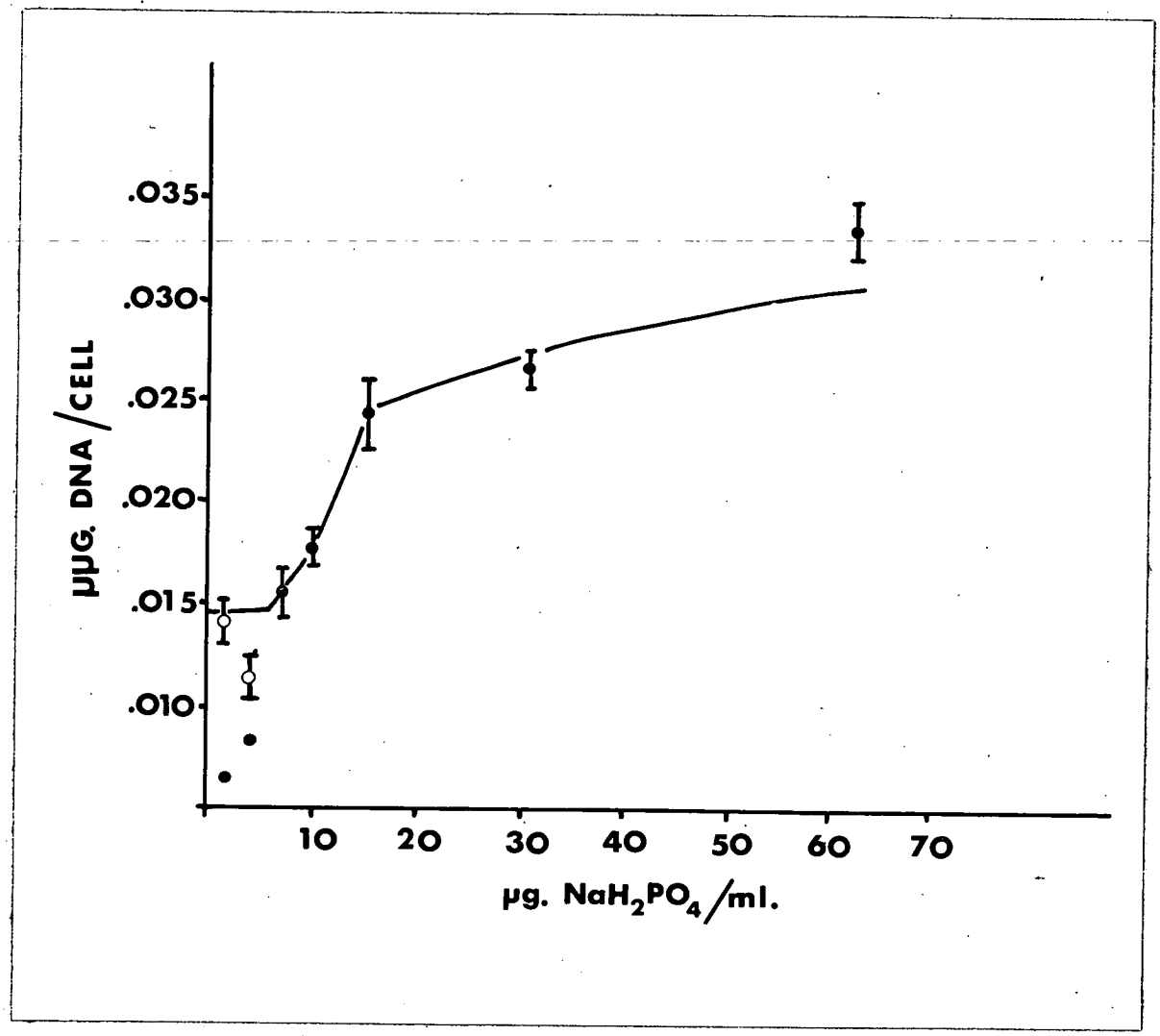

air flow
from
Hyflo pump
Entry of medium
delivery head
Culture
vessel
Reservoir
12 in.
stirrer
Constant head vessel
Collecting
vessel
Pump

cell concentrations no factor was limiting, with the result that the cell generation time exceeded the rate of elution from the culture. The net effect was that the cells increased in concentration until the level of phosphate became limiting, at which time the rate of growth of the cells decreased to equal the rate of entry of fresh medium to the culture, producing a steady state.

Results and Discussion

Cultures of S. pombe were grown in the basic Edinburgh Minimal Medium, but with varying concentrations of sodium dihydrogen orthophosphate, to see what effect different levels of phosphate might have on the mean amount of DNA per stationary phase cell. Figure 18 shows the relationship between the initial concentration of NaH_2PO_4 in the culture medium and the mean DNA content per stationary phase cell. It can be seen that there is a relationship between the two such that at low concentrations of phosphate the cells enter stationary phase with the 1c (0.015 µµg. DNA/cell) amount of DNA per cell, whereas at high concentrations of phosphate stationary phase cells stop growing in G2. Between these two extremes the stationary phase population is a mixture of cells, the ratio of G1 to G2 depending on the initial amount of phosphate present in the medium. At the lower levels of phosphate concentration

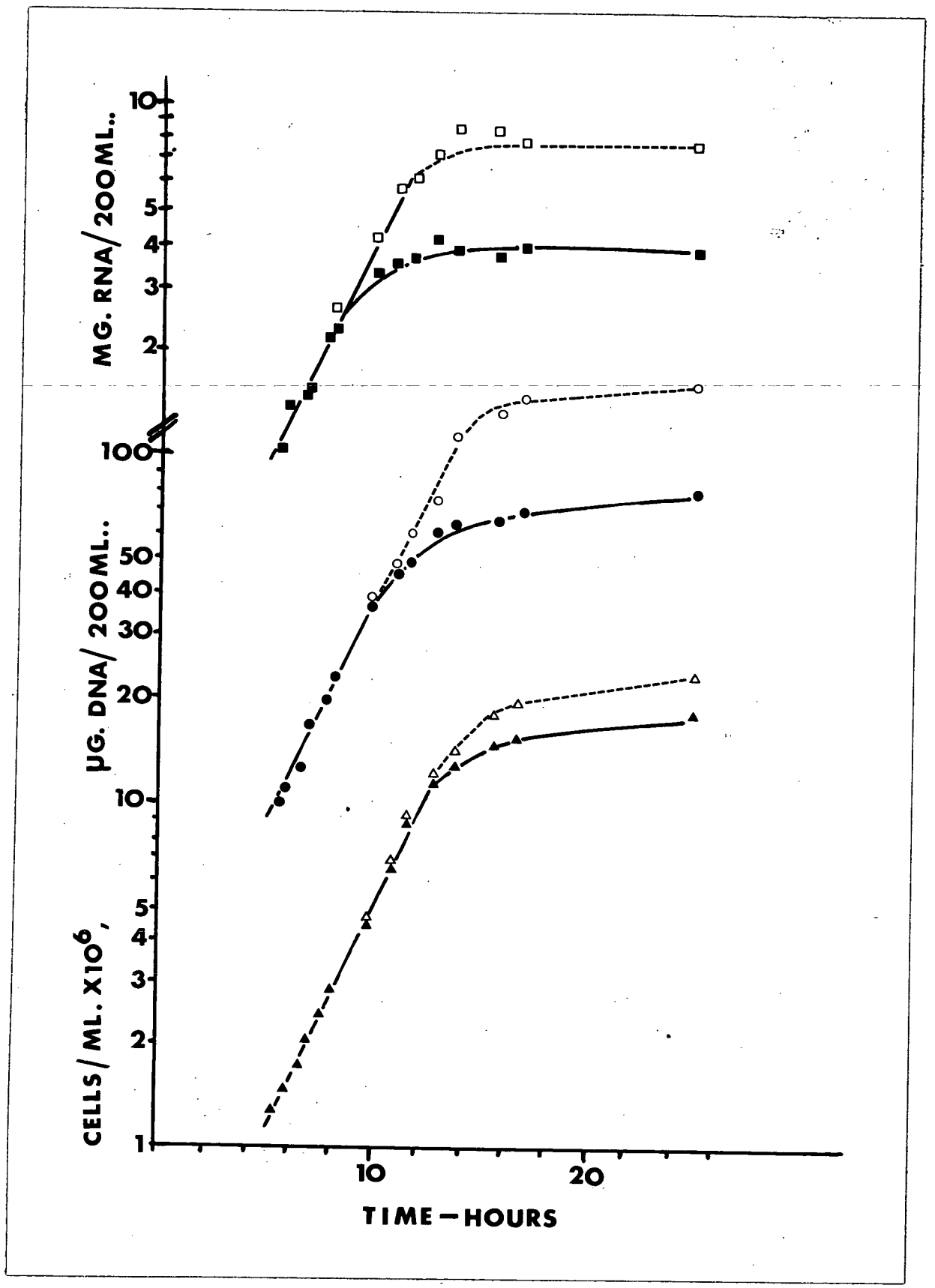
Figure 18. Variations in the amount of DNA per stationary phase cell with changes in the initial phosphate concentration in the medium. Solid circles represent the means of eight estimates and the bars their standard errors. The open circles represent the corrected values for DNA content after allowing for non-viability in the phosphate starved cultures.


.035
.030
.025
.020
.015
.010
µµG. DNA /CELL
10
20
30
40
50
60
70
µg. NaH2PO4/ml.

the measured mean DNA content of cells is considerably lower than the 1c value, but these phosphate starved populations quickly lose viability. If this is taken into account, with the assumption that non-viable cells undergo autolysis resulting in degradation and loss of DNA, then the mean DNA content of these starved cells approximates to the 1c value.

In order to investigate the effect that the level of phosphate was having during exponential growth and entry of the culture into stationary phase, cultures were inoculated in EMM 1 and allowed to grow to early log. phase. When the culture had reached a cell concentration of about 3×10^6 cells/ml. it was split in two and NaH_2PO_4 was added to one half to a concentration of 62 µg./ml. Both cultures were followed in terms of cell concentration, DNA, RNA, and the phosphate content of the cold 5% PCA soluble and insoluble fractions. Figure 19 shows the increase in cell number and the DNA and RNA content per 200 ml. sample. There is an exponential increase in all three parameters up to about 3×10^6 cells/ml., but after this point in the EMM 1 culture the rate of increase of both nucleic acids and, to a lesser extent, cell number begins to fall off. The shapes of the DNA and RNA curves are essentially similar, but the relatively greater increase in the number of cells per ml. accounts for the drop observed in the mean DNA and RNA content of cells as they enter stationary phase. These parameters are plotted as a function of the cell

Figure 19. RNA per sample (squares), DNA per sample (circles) and cell concentration (triangles) in an EMM 1 culture (solid symbols) and an EMM 1 culture with added phosphate (open symbols).


MG. RNA/200ML..
10
5
4
3
2
100
50
40
30
20
µG. DNA/200ML..
10
5
4
3
2
1
CELLS/ML. X10⁶,
10
20
TIME—HOURS

concentration in figure 20, where it can be seen that in the high phosphate cultures RNA per cell is roughly halved as cells enter stationary phase, whereas the DNA per cell drops only marginally towards the 2c value. The latter contrasts markedly from the culture grown in EMM 1, where the normal drop in the mean DNA content per cell begins at a cell concentration of about 4 - 5 x 10^6 cells/ml.

Figure 21 shows the increase of both acid soluble pool phosphate and macromolecular phosphate in the two cultures. There is a striking difference between the phosphate content of the pool in EMM 1 cultures and that of the culture with added phosphate. The decrease in the total cold acid soluble phosphate coincides with the falling off in the rate of synthesis of phosphate containing macromolecules, DNA and RNA. In EMM 1 cultures there is very little phosphate containing material in the pool as the cells enter stationary phase, whereas in the increased phosphate medium, stationary phase cells have a considerable pool of low molecular weight phosphate compounds.

The level of phosphate is therefore having a considerable effect on the synthesis of nucleic acids in these cells. Over the range of concentrations used in the preceding experiments it is determining whether the cells will enter stationary phase in the 1c or 2c state. In view of this it was decided to set up a continuous culture of S. pombe in a chemostat under conditions of limiting phosphate. During normal exponential

Figure 20. Variations in the RNA (squares) and DNA (circles) contents of cells in EMM 1 (solid symbols) or EMM 1 with added phosphate (open symbols) as a function of the cell concentration.

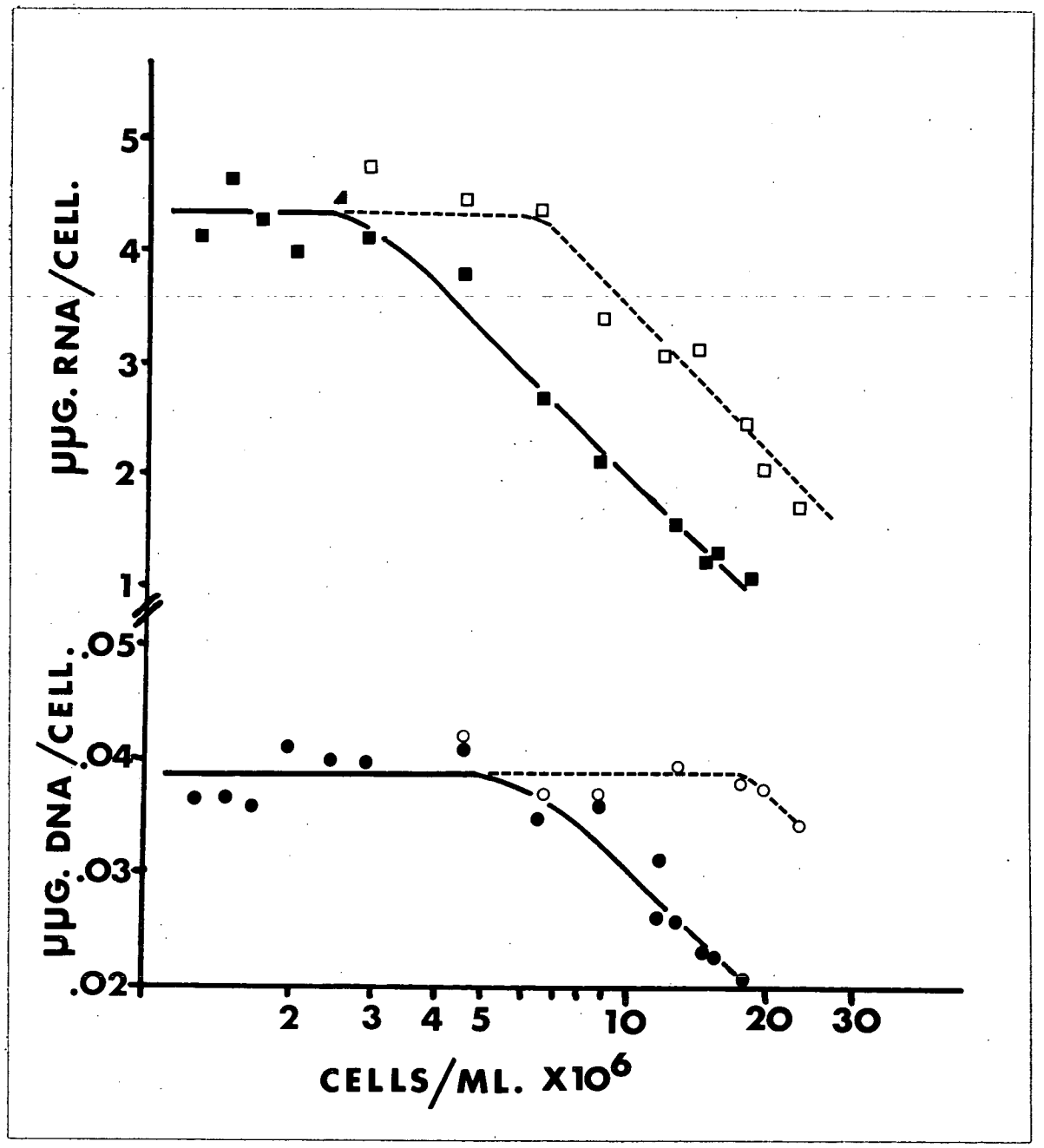
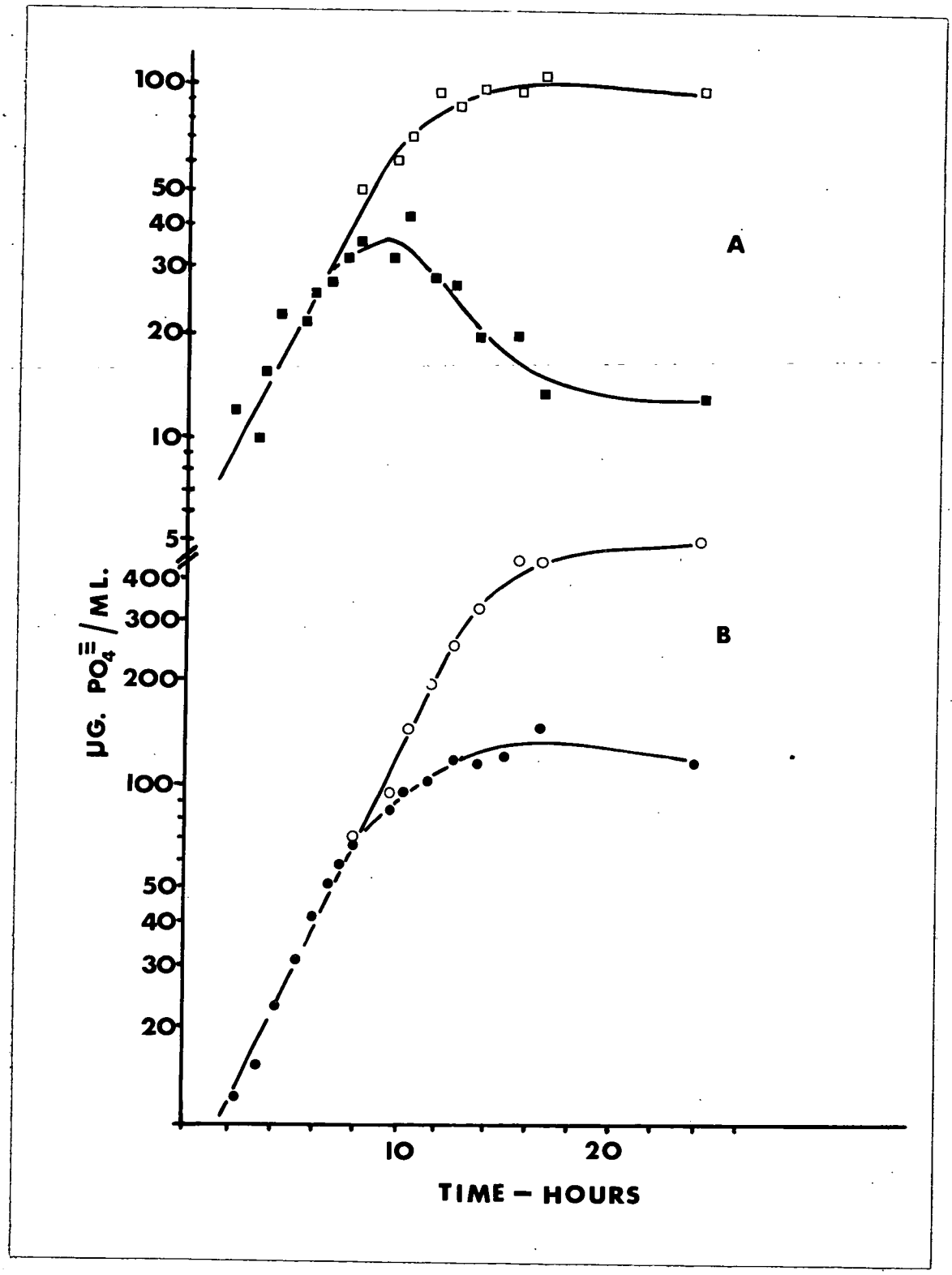

μμG. RNA/CELL.
5
4
3
2
1
μμG. DNA/CELL.
.05
.04
.03
.02
2
3
4
5
10
20
30
CELLS/ML. X10⁶

Figure 21. Cold acid soluble phosphate (A) and incorporated phosphate (B) in cells grown on EMM 1 (solid symbols) or EMM 1 with added phosphate (open symbols).


100
50
40
30
20
10
5
400
300
200
100
50
40
30
20
A
B
μG. $PO_4^{\equiv}$ / ML.
10
20
TIME – HOURS

growth in EMM 1 the culture medium is depleted of phosphate at about 3 x 10^6 cells/ml., and yet growth in terms of cell concentration continues for about three cell generations. There are, therefore, considerable reserves of phosphate within the cell, and it is necessary to severely limit the amount of phosphate in the medium in order to attain a stable continuous culture. After a few trials it was found that a concentration of 3 µg. NaH_2PO_4/ml., with a flow rate of one culture volume per 24 hours, yielded a stable culture with a cell concentration of about 2.5 x 10^6 cells/ml.

Figure 22 shows the growth of the culture with time. The cell concentration remains constant indicating that the cells are growing with a mean generation time of 24 hours. Despite the constancy of cell number the culture could not have been in balanced growth, because the optical density of the culture increased steadily over the course of the experiment. This increase suggested that in some way the processes of growth had been dissociated from division and that the cells were increasing in size. This was confirmed while the cells were observed under the microscope during the course of cell counting. Not only did the cells become longer, but they also began to branch in one or more places. Figure 23 shows a typical branched cell from one of these low phosphate continuous cultures. Another peculiar phenomenon of these branched cells, which can also be seen in figure 23, is the fact that they

Figure 22. Cell concentration (open circles) and optical density (solid circles) in a continuous culture in EMM under conditions of limiting phosphate.

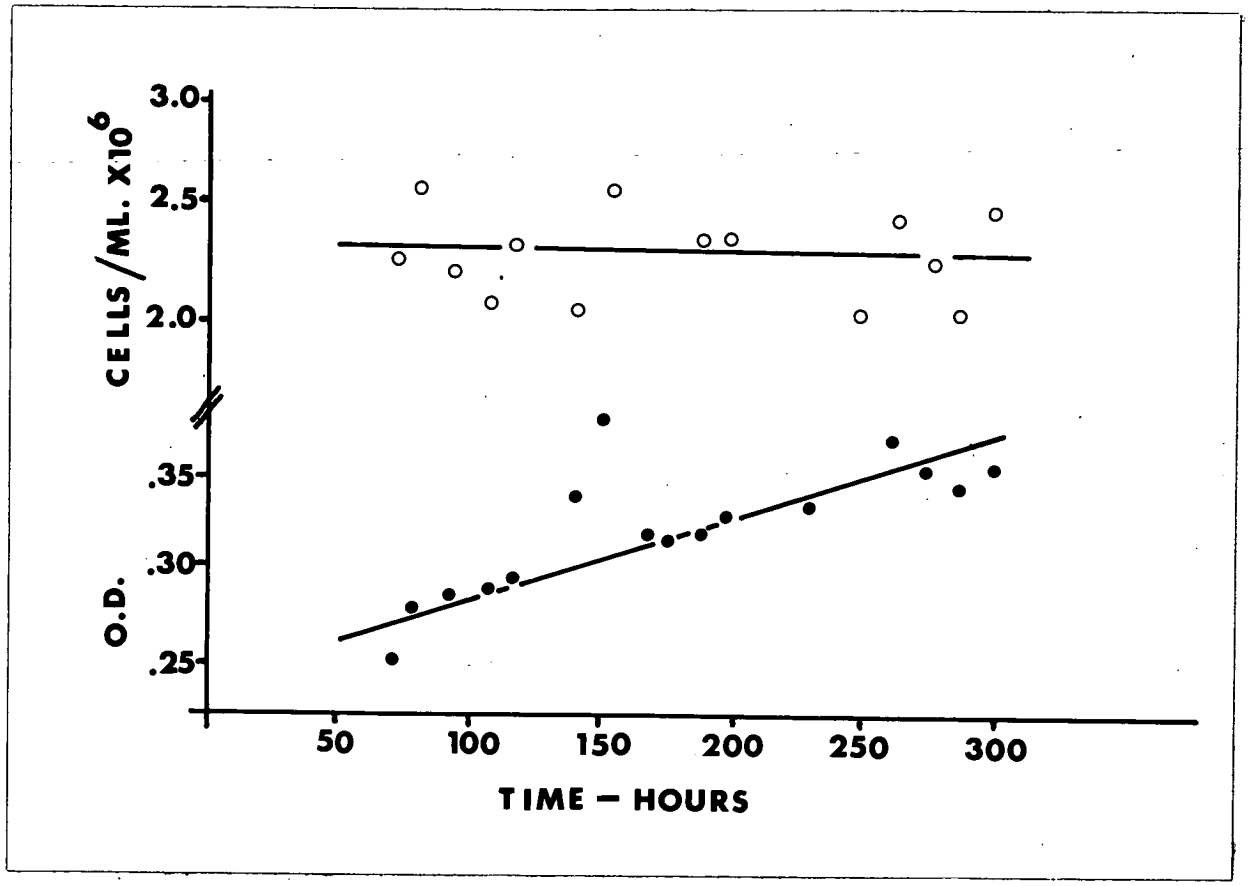
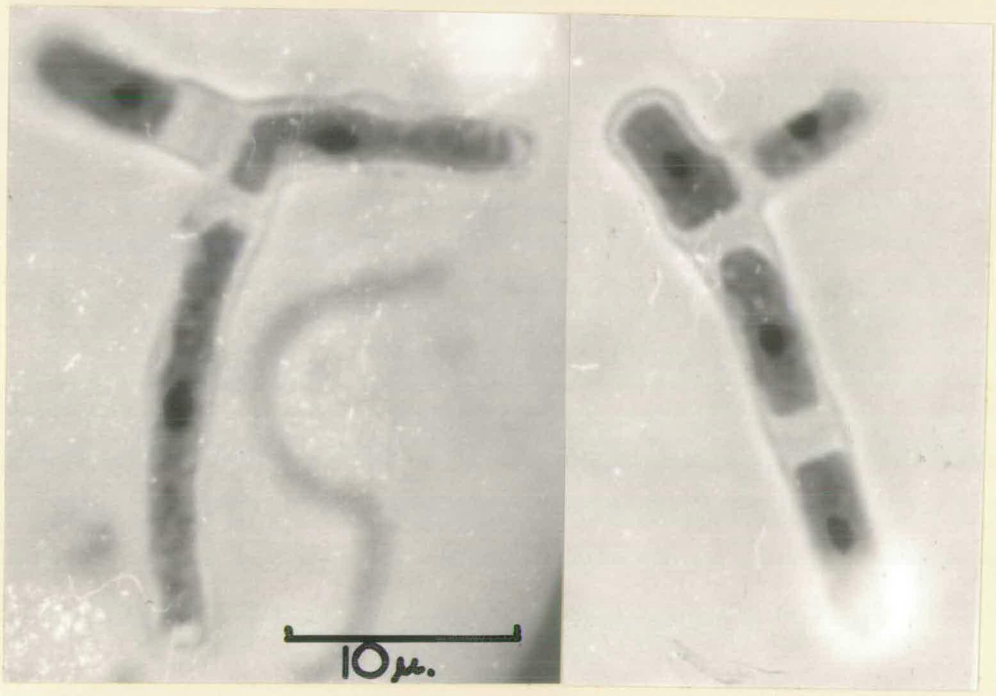

3.0
2.5
2.0
CELLS/ML. X10^6
.35
.30
.25
O.D.
50
100
150
200
250
300
TIME — HOURS

Figure 23. Photographs of Giemsa stained cells from a 200 hour continuous culture under conditions of limiting phosphate. The cells are branched and contain several nuclei.


15 µm.

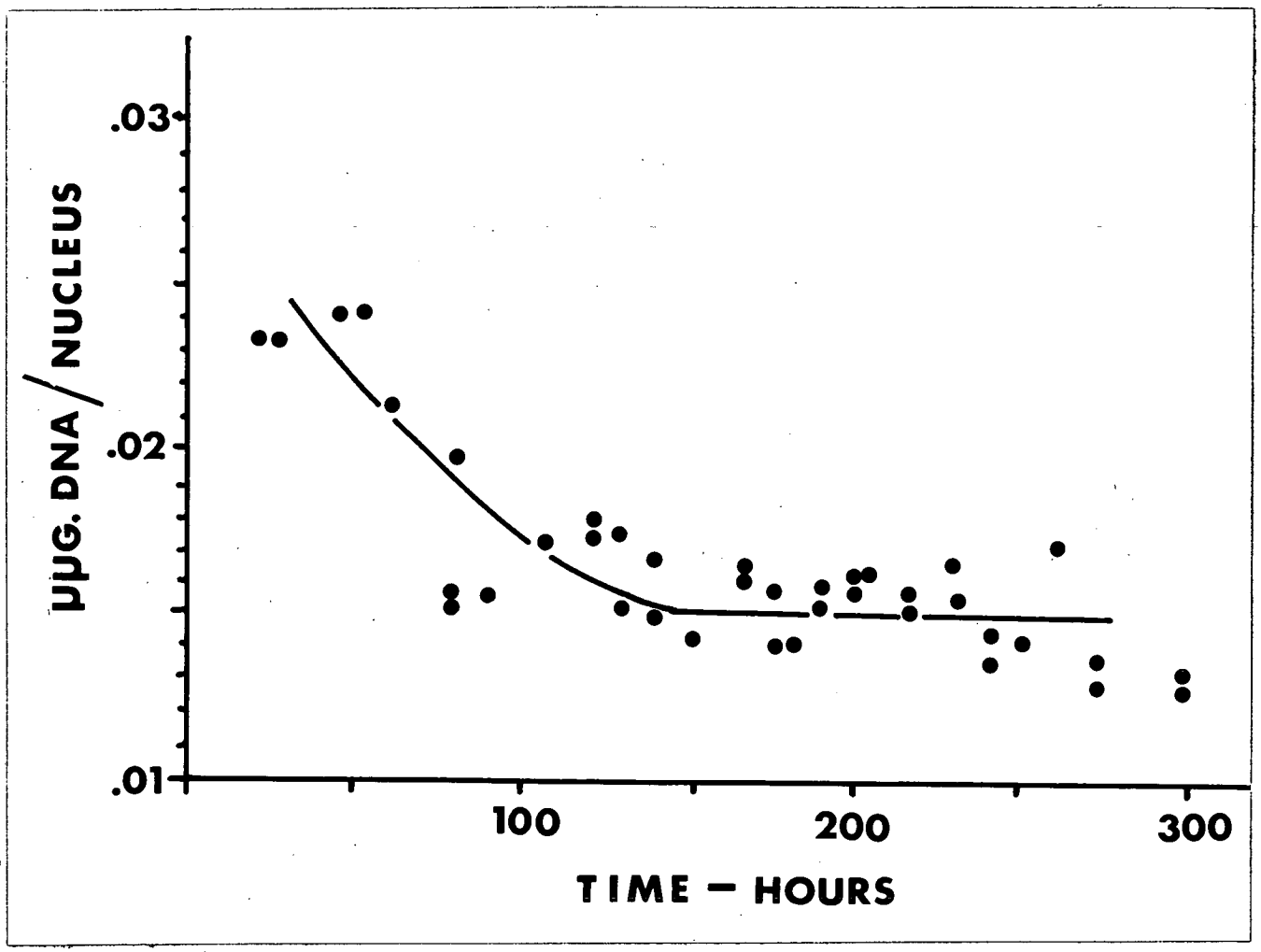
possess, in some cases, several nuclei which are not separated by cell plates. Counts made on Giemsa stained slides showed that the average number of nuclei per cell increased with time of culturing and was roughly proportional to the increase in optical density (see table 4). Because of this the DNA content of nuclei was calculated rather than the amount of DNA per cell. The total DNA in the culture rose in a similar fashion to optical density with the result that the amount of DNA per nucleus remained stable at about the 1c value (figure 24).

The possibility existed, because the mean DNA content per nucleus was so low, that some of the cells containing nuclei were inviable and that loss of DNA from the nuclei might have occurred, in much the same way as there was death in the phosphate starved stationary phase cultures. This was unlikely in view of the fact that the estimate of DNA per nucleus was made on the basis of Giemsa stained nuclear counts. The Giemsa stain, after the extraction procedure, will only react with DNA and the residual RNA. If there was sufficient DNA material to yield a clearly stained nucleus it would also be measured by the diphenylamine reaction. The residual RNA remaining after the RNase treatment would not yield sufficient colour to show up the nuclei clearly as was shown by DNase digested controls of some slides. Nevertheless, viability counts were made and it was confirmed that the cultures were between 95 and 100%

Table 4. Average number of nuclei per cell in the continuous culture experiment.

Time (hours)	Cells/ml. x 10^{-6}	O.D.	Nuclei/cell
81	2.58	.275	1.17
141	2.03	.340	1.22
189	2.23	.317	1.35
228	2.03	.379	1.43
274	2.25	.351	1.62
300	2.48	.355	1.52

Figure 24. The amount of DNA per nucleus in continuously cultured cells of S. pombe as a function of the length of culturing.

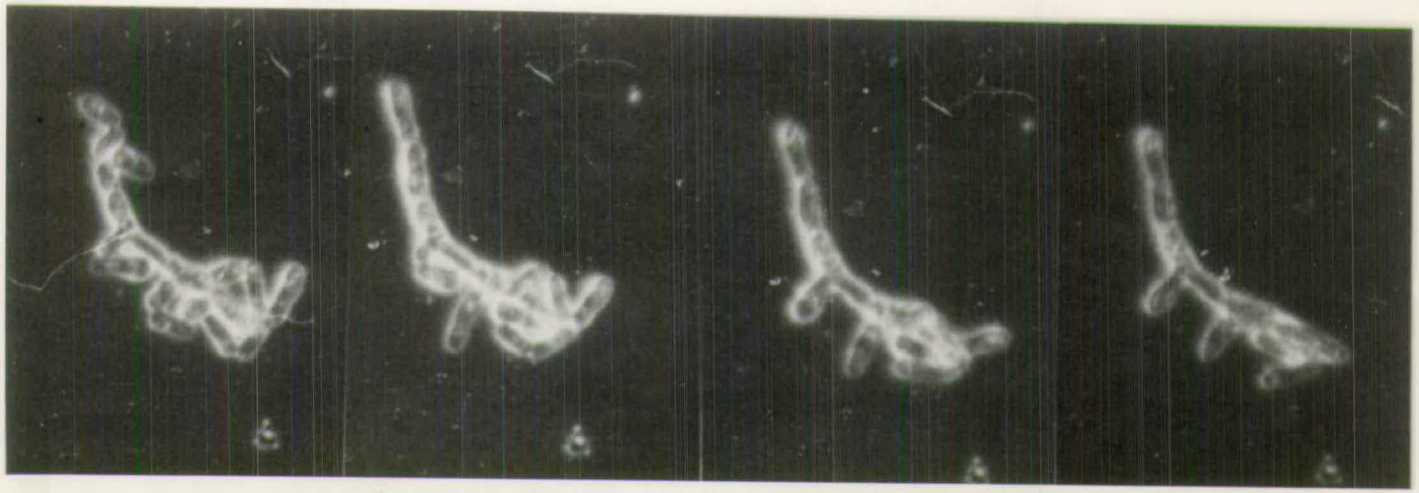

.03
.02
.01
μμG. DNA / NUCLEUS
100
200
300
TIME — HOURS

viable. The long branched cells were also viable, see figure 25, for after plating on fresh EMM 2 agar they immediately began to divide into several cells, the number possibly reflecting the number of nuclei present in the original cell.

It was hoped to make synchronous cultures from the limiting phosphate continuous cultures, but for technical and practical reasons this proved impossible. The major factor was the heterogeneity in the cell cultures. For normal synchronous cultures cells are selected by the sucrose gradient according to size, and, because there is a relationship between cell size and the stage that the cell is in the cell cycle, the culture divides synchronously for a few generations. In the continuous cultures there was a tremendous variation in the size of cells, with the extremes being multinucleate, so the basis of the synchronisation procedure no longer held. Secondly, because at least 10 litres of culture are required to produce a synchronous culture for DNA assay, the technicalities in making such a culture were immense and not possible.

The results from the phosphate experiments still do not allow any definitive description of the state of the cells in stationary phase. The two possibilities still remain, namely, that the stationary phase is either a mixture of 1c and 2c cells, or that it is a homogeneous population of cells in mid S phase. The results do, however, give more credence to the first of the alternatives.

Figure 25. A series of time lapse photographs of a branched cell from a limiting phosphate continuous culture after plating on to EMM 2 agar. The cells were photographed under dark field illumination and each photograph represents an interval of one hour.



The amount of phosphate in the medium which is required to give a homogeneous 2c stationary phase population is roughly five times as much as that required to produce a homogeneous 1c stationary population. It is difficult to conceive of any control mechanism that would exercise such a fine regulation of the point at which the synthesis of DNA would stop over such a wide range of starting phosphate concentrations in the medium. However, if DNA synthesis is an all or nothing phenomenon, with the cells stopping growth in either G1 or G2, as appears to be the case in other cell types (e.g. stationary phase G1 cells, Williamson and Scopes, 1961, Stonehill and Hutchinson, 1966, Prescott and Carrier, 1964, and Kimball and Vogt-Kohne, 1961; stationary phase G2 cells, MacDonald, 1958; and mixed populations of G1 and G2 cells, reviewed in Gelfant, 1963), the problem is slightly different. It changes from a problem of maintenance of DNA synthesis to one of the initiation of DNA synthesis.

Though these two processes are intimately related there are essentially three conditions that must be met before the transition from G1 to S can be accomplished. There must be an adequate supply of polymerases and deoxyribonucleotide triphosphates and the DNA must be in the template condition. Phosphate molecules are intimately involved in the second of these processes, namely, the supply of energy and the building blocks for the formation of the new DNA polymer. When the supply of

phosphate becomes limiting there will be competition for the available amount not only between those synthetic pathways that produce phosphate containing macromolecules, but also between the metabolic pathways that require energy in the form of adenosine triphosphate. If the level of phosphate within the cell during stationary phase was too low to allow the production of deoxyribonucleotide triphosphates, and let the synthesis of other macromolecular components continue, e.g. RNA turnover (Mitchison, pers. commun.), then the level of phosphate in the medium could ultimately determine whether the S phase would be initiated or not.

Another point in support of the mixed 1c and 2c stationary phase population hypothesis comes from the continuous culture experiments. In these cultures the mean amount of DNA per nucleus was essentially the same as that of a 1c nucleus. As this is a mean value it indicates that, under these growth conditions, the cell cycle is composed mainly of G1, and that the S phase must be fairly close to nuclear division. If the various phases of the cell cycle had been extended to occupy a 24 hour cell cycle, most of the nuclei, as in the normal cultures, would be observed to be in G2 and the mean amount of DNA per nucleus would approach the 2c value. If the effect of the low phosphate was to lengthen the S phase, and to make it occupy the majority of the cell cycle, then the mean DNA content of nuclei would be 1½c. Neither of the latter two

explanations fit the experimental results, which support the idea of an extended G1. The conditions of limiting phosphate in the continuous culture are similar to the conditions present as the cells enter stationary phase in the low phosphate media. If DNA synthesis is delayed such that the S phase is well after fission in the former, there seems no reason why this should not also happen as the cells enter stationary phase.

Clearly the DNA synthesis pattern can be altered by variations in the phosphate content of the medium, but it is unfortunate that this change in the cell cycle could not be used to study the effect of such a change on the other macromolecular synthetic patterns in the cell cycle. As a result, other environmental changes were made in an attempt to produce a system which could be fully analysed.

Chapter 8

The Effect of Increased Generation Time on the DNA Synthesis Cycle

Change in the Carbon Source

Table 5 summarises the carbon sources that S. pombe will and will not grow on. Those that do support growth of this organism all do so at very similar growth rates of between 2½ and 3½ hour generation times. The differences between these was not considered sufficient to bring about possible alterations to the DNA synthesis cycle that could be picked up by biochemical assays on exponentially growing random cultures. Because of this it was decided to try and isolate mutants, produced in the presence of N-methyl-N-nitroso-N'-nitroguanidine or after ultraviolet irradiation, that would grow on one or more of these carbon sources. The following potential carbon sources were tested: lactose, acetate, ethanol, tryptone, pyruvate and succinate, and, in addition, glycerol was also tested because some yeasts can metabolise this as a carbon source (Ingram, 1955). All the attempts to isolate mutants capable of growing on different carbon sources were unsuccessful, but it was found that S. pombe would grow on EMM 2 with the glucose replaced by 3% glycerol.

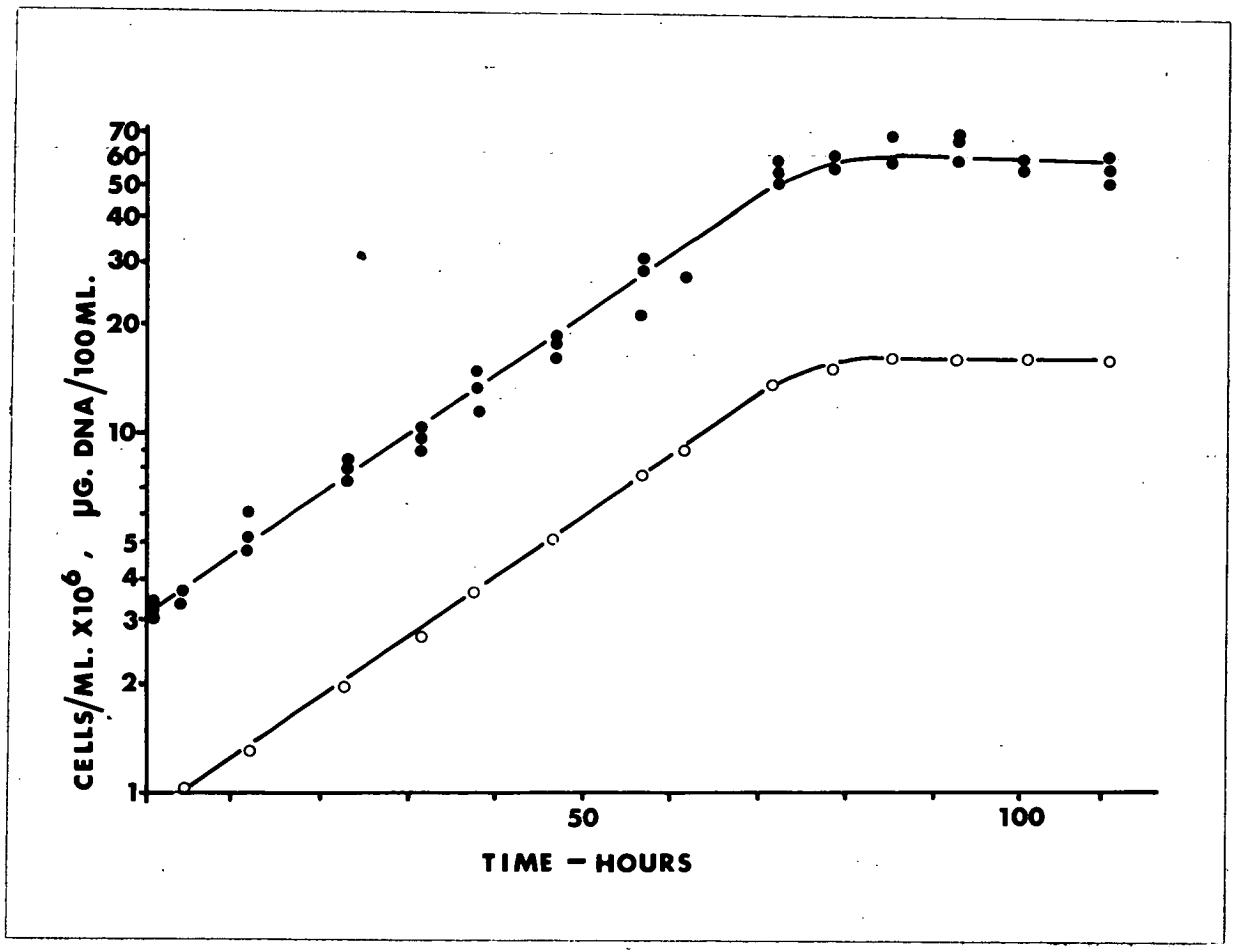
Figure 26 shows the growth of S. pombe in EMM 2 with 3%

Table 5.

Carbon Sources that:	
will support growth	will not support growth
Glucose	Lactose
Sucrose	Galactose
Maltose	Trehalose
Fructose	Lactate
Mannose	Succinate
Raffinose	Citrate
Cellobiose	Acetate
	Pyruvate
	Acetaldehyde
	Ethanol
	Tartrate
	Peptone
	Tryptone
	Mannitol

From Mitchison (pers. commun.) and Ingram (1955)

Figure 26. Cell concentration (open circles) and DNA per sample (solid circles) in a culture grown on EMM 2 when glycerol replaced glucose as the carbon source.


70
60
50
40
30
20
10
5
4
3
2
1
CELLS/ML. X10⁶, µG. DNA/100 ML.
50
100
TIME – HOURS

glycerol replacing the glucose. The mean generation time in this medium was 18 hours at 32°C so that if a relationship had existed between the increase in generation time and a disproportionately long G1 it should have shown up in mass culture under these conditions. In fact the rate of increase of DNA exactly paralleled that of the cell concentration, even during the entry of cells into stationary phase, though the final yield of cells on this medium is much lower than on normal EMM 1 or 2. Throughout the whole growth of the culture the mean DNA content per cell remained constant at 0.0386 μμg. DNA/cell (s.e. of mean = 0.0007). This value is not significantly different from the mean DNA content of normal log. phase cells, which indicates that the increase in the generation time due to a change in the carbon source has extended each cell cycle phase proportionately.

One surprising feature of these cultures, compared to the normal cultures, was the lack of a drop in the mean DNA content of cells as they entered stationary phase. This can be explained in two ways. Firstly the cells go into stationary phase at a much lower cell concentration, presumably because glycerol is not as efficient as an energy source as the sugars. In normal EMM 2 cultures at this concentration cells still contain the characteristic log. phase complement of DNA (see figure 20). Secondly this value drops to the 2c value characteristic of EMM 2 stationary phase slowly over a period

of three days. If the increase in the generation time is taken into account in these glycerol cultures then it would take up to three weeks before the cells could be said to be in stationary phase and equivalent to the three day EMM 2 cultures. In fact the cells begin to lose viability before this, which, coupled with the risk of bacterial contamination over these long periods of time, makes the analysis of the entry into stationary phase impractical in these cultures. However, this is a minor aspect and clearly the change of carbon source is having no differential effect on the length of the G1 phase in this organism, which is detectable by the methods used.

DNA Synthesis Cycle of Schizosaccharomyces pombe at 17°C

Having been unable to produce a change in the DNA synthesis cycle by an increased generation time due to a change in the carbon source the effect of temperature was investigated. As discussed in the introduction to this section the most promising temperature at which to look for alterations to the DNA synthesis cycle was 17°C.

A bulk culture was grown up from a small inoculum in EMM 2 at 32°C and sampling was started when the culture reached a cell concentration of 1 x 10^6 cells/ml. After three hours the culture was split in two, one half being cultured at 32°C, the other half being quickly cooled to 17°C. The growth of both

cultures was then followed in terms of the increase in cell concentration and DNA. Figure 27 shows the results of one of these experiments. On being cooled to 17°C the generation time of S. pombe was increased to 15 hours, but this increased further once the cells had reached a cell concentration of 10×10^6/ml. The mean amount of DNA per cell is shown in figure 28, where it is plotted against the cell concentration. From this it appears as though there was a decrease in the mean DNA content of log. phase cells at the lower temperature, but it will also be seen that there was a similar drop in the control culture. There is no significant difference between the overall mean of the 17°C culture, 0.0330 μμg. DNA/cell (s.e. = 0.0004), and that for the control cultures, 0.0341 μμg. DNA/cell (s.e. = 0.00075).

Despite the lack of significant difference between the experimental and control cultures, this decrease was of the order to be expected if a shift of the S period had been brought about. In view of this, it was decided to see what happened in the lag phase of these low temperature cultures. If there was an extended G1 phase being inserted between nuclear division and the S phase it should be readily observable as the cells pass out of the lag phase into exponential growth.

Figures 29 and 30 show the results of such an experiment. Essentially the pattern of DNA synthesis was the same as that in the 32°C lag phase culture. The lag phase lasted for about

Figure 27. Cell concentration (open symbols) and DNA per sample (solid symbols) in an EMM 2 culture divided and cultured at 32°C (circles) and 17°C (squares).

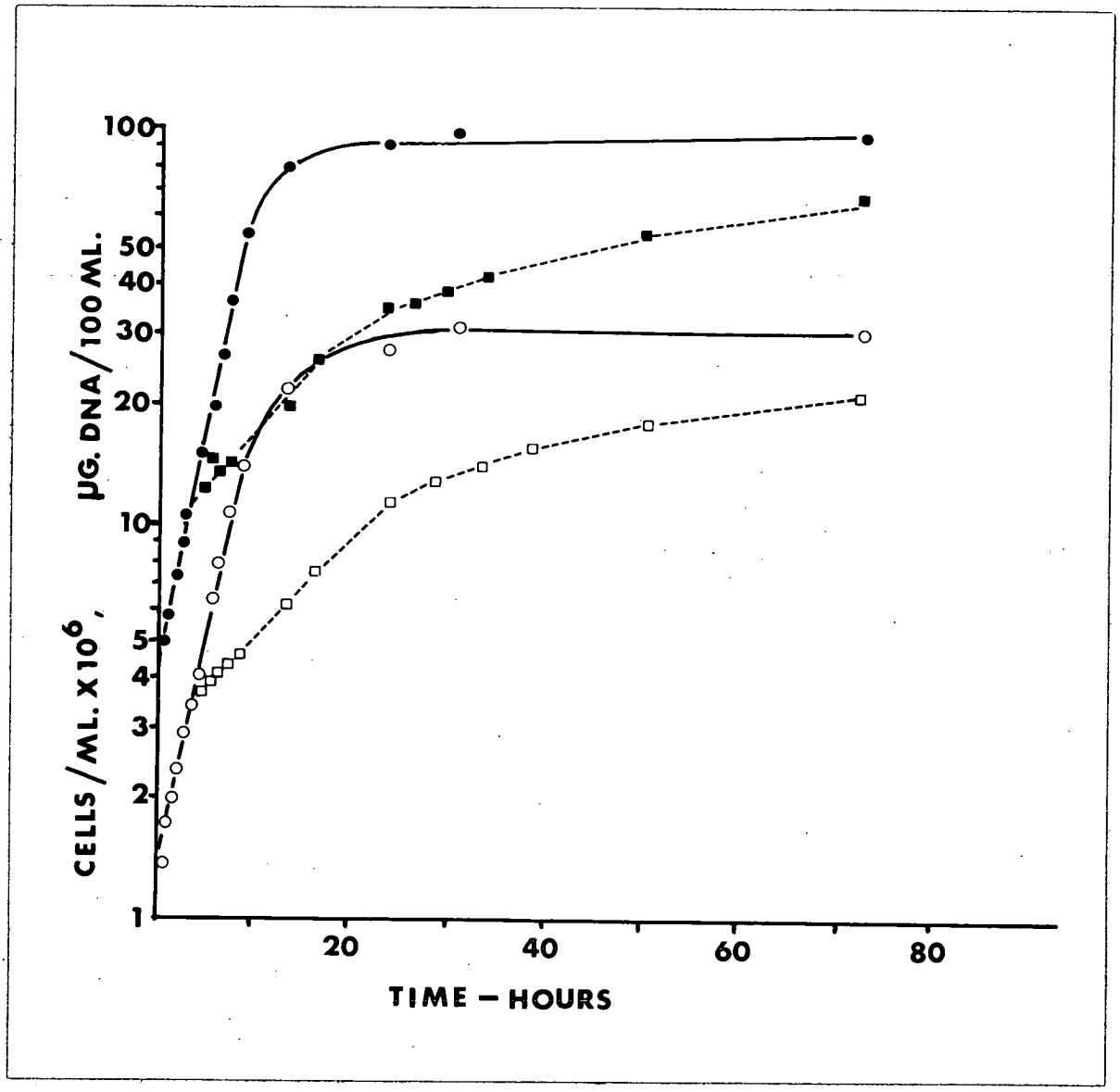

100
50
40
30
20
10
5
4
3
2
1
CELLS/ML. X10^6, µG. DNA/100 ML.
20
40
60
80
TIME — HOURS

Figure 28. Variations in the amount of DNA per cell with increasing cell concentration in EMM 2 at 17°C (open circles) and 32°C (solid circles). The bars represent the standard errors of the means derived from three experiments.

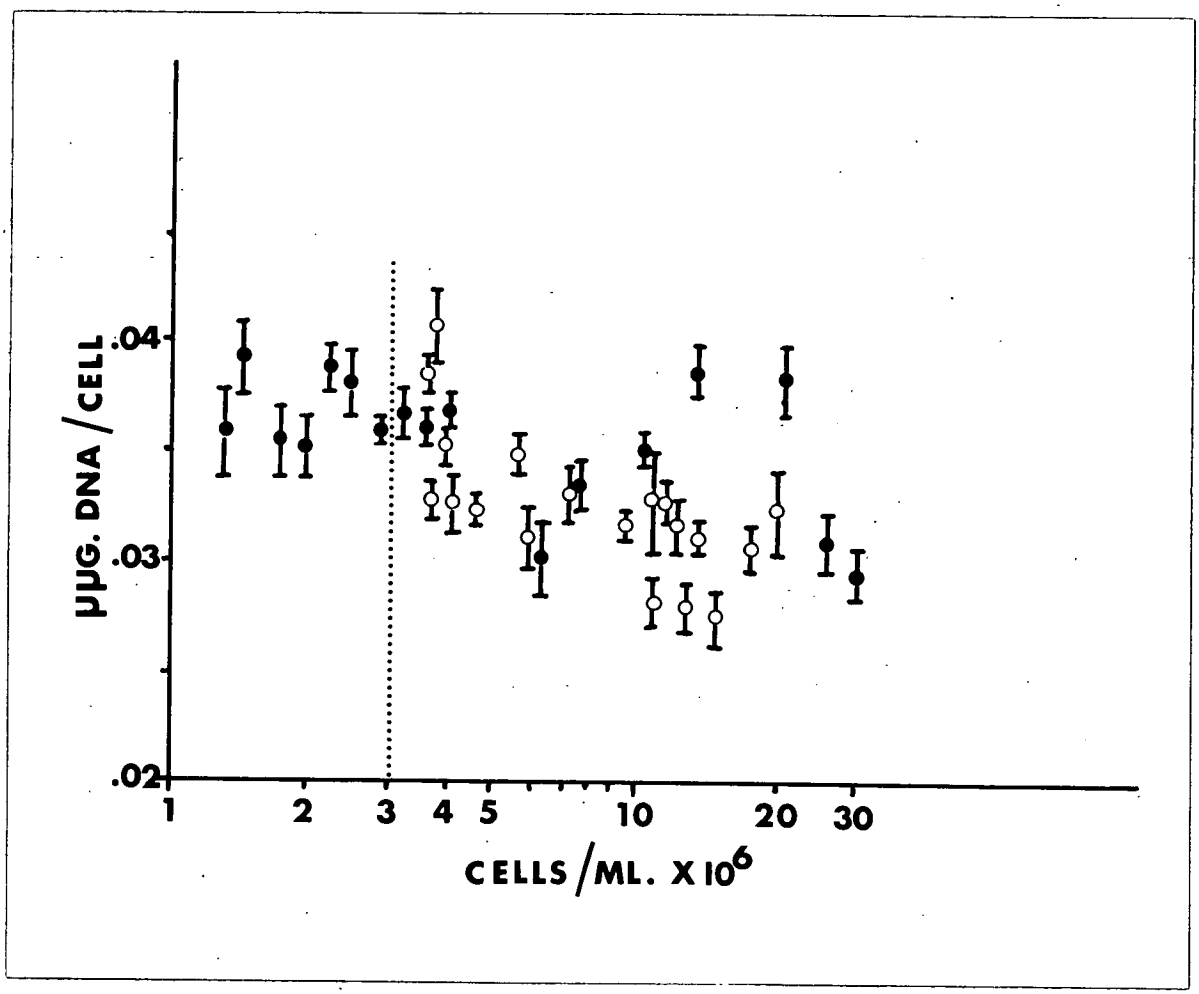

μμG. DNA /CELL
.04
.03
.02
1
2
3
4
5
10
20
30
CELLS /ML. X 10^6

Figure 29. DNA per sample (solid circles), cell concentration (open circles) and cell plate index (triangles) following inoculation at 17°C in EMM 2.

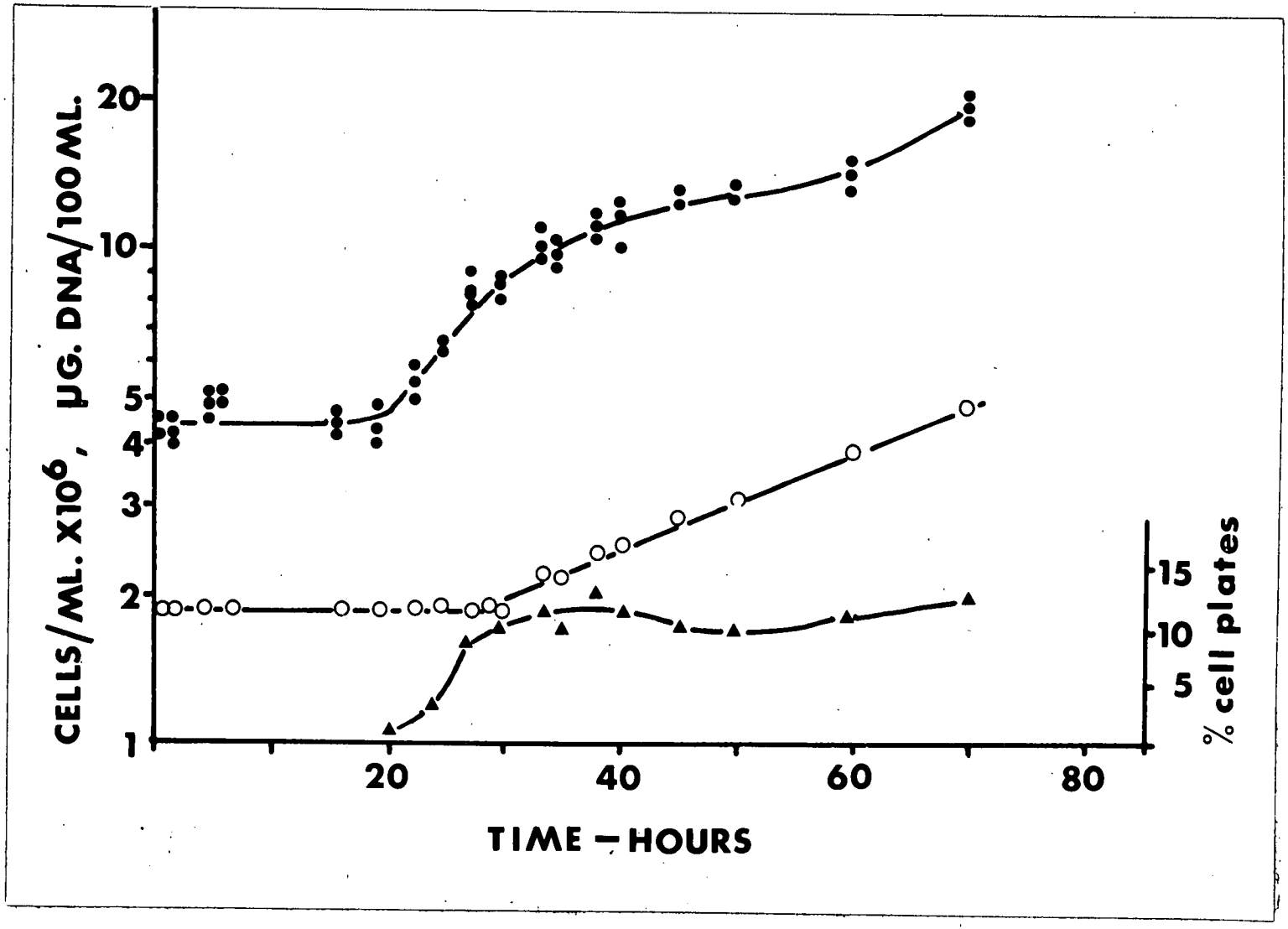
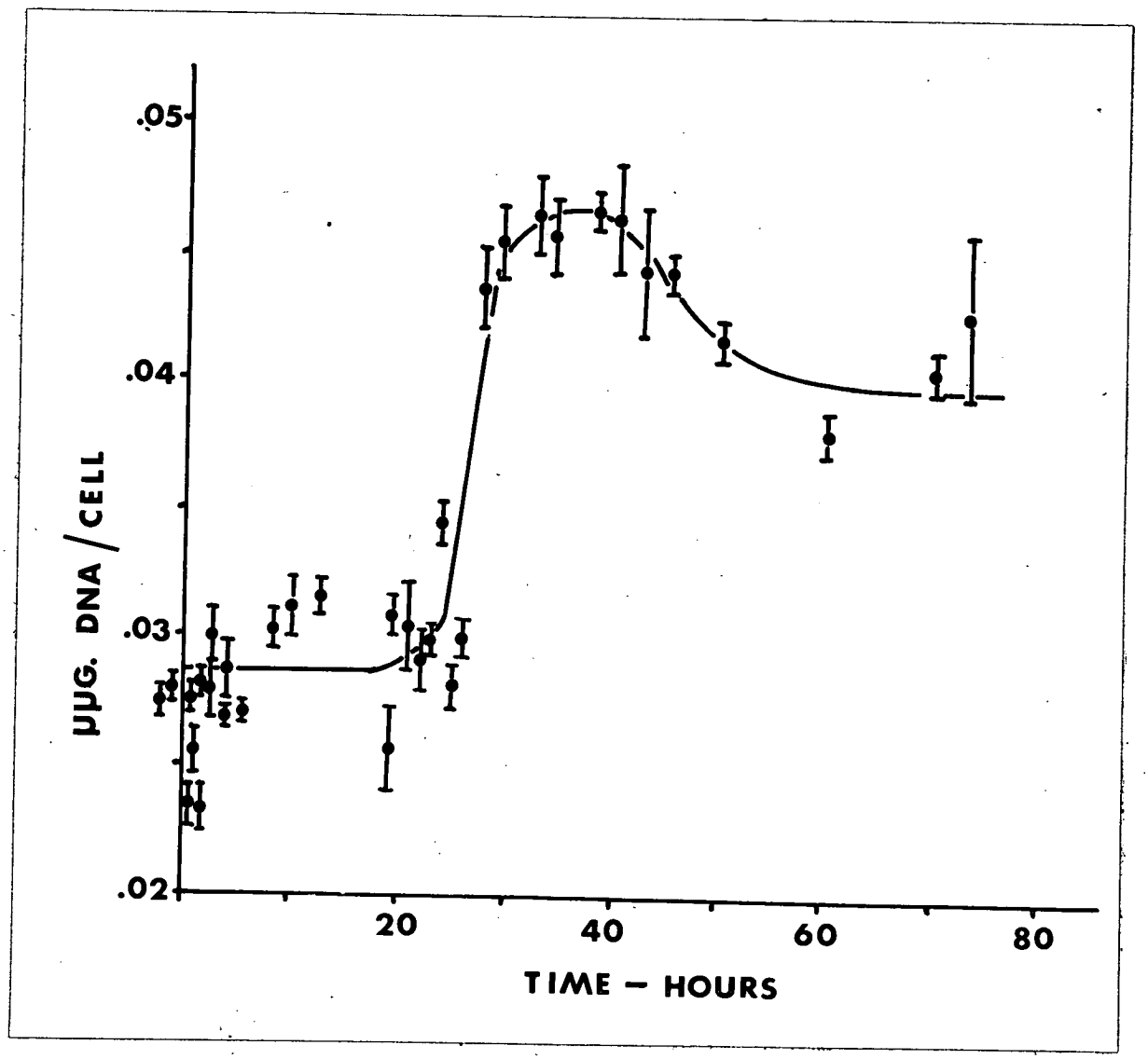

CELLS/ML. X10^6, µG. DNA/100 ML.
20
10
5
4
3
2
1
20
40
60
80
TIME – HOURS
15
10
5
% cell plates

Figure 30. Changes in the mean DNA content of cells following inoculation at 17°C in EMM 2. The solid circles represent the means of two experiments and the bars their standard errors.


.05
.04
.03
.02
μμG. DNA / CELL
20
40
60
80
TIME – HOURS

30 hours after which there was an increase in the cell concentration. This rise was preceded by a rise in the cell plate index, which was itself preceded by a rise in the amount of DNA per ml. of culture. There was no rise in the amount of DNA per ml. of culture following inoculation, as there was in the EMM 1 lag phase cultures, because these experiments were performed in EMM 2 where cells were already in G2 before inoculation.

In figure 30 are the combined results of two experiments which show the mean DNA content of cells as they progressed from the time of inoculation, through lag phase, and into the phase of exponential growth. Although there is considerable variation in the points in the lag phase the overall mean of these is 0.0280 μμg. DNA/cell (s.e. of mean = 0.0005), which approximates to the 2c value. This remained constant up to a time just prior to the first appearance of cell plates, when it increased sharply towards the 4c value. This peak is somewhat broader than might be expected, but this is essentially a product of the combination of two separate experiments, in which the positions of the increase in the cell plate index and the amount of DNA per cell were slightly different.

It therefore appears that the DNA synthesis cycle of S. pombe is not susceptible to differential changes in the lengths of the various cell cycle phases as a result of increases in the generation time due to temperature or

'nutritional' changes. This contrasts with the work done on other cell types, but, with the exception of the bacteria, all the cell types in which these phenomena have been studied have a large proportion of their normal cell cycle occupied by a G1 phase. It may be that, in eukaryotic cells that have no G1, the preparations for the start of S phase are carried out in the preceding G2, with the result that the S phase can commence immediately after nuclear division. It is only when some restraint, which is directly involved in the initiation of DNA replication, is applied to the cell that the two phenomena can be dissociated. The level of phosphate, which, though not specific to DNA synthesis, is intimately concerned with it, has this effect when it becomes limiting. Another, and more specific, way of dissociating nuclear division and DNA synthesis is by the use of inhibitors. It is this which is the subject of the following section.

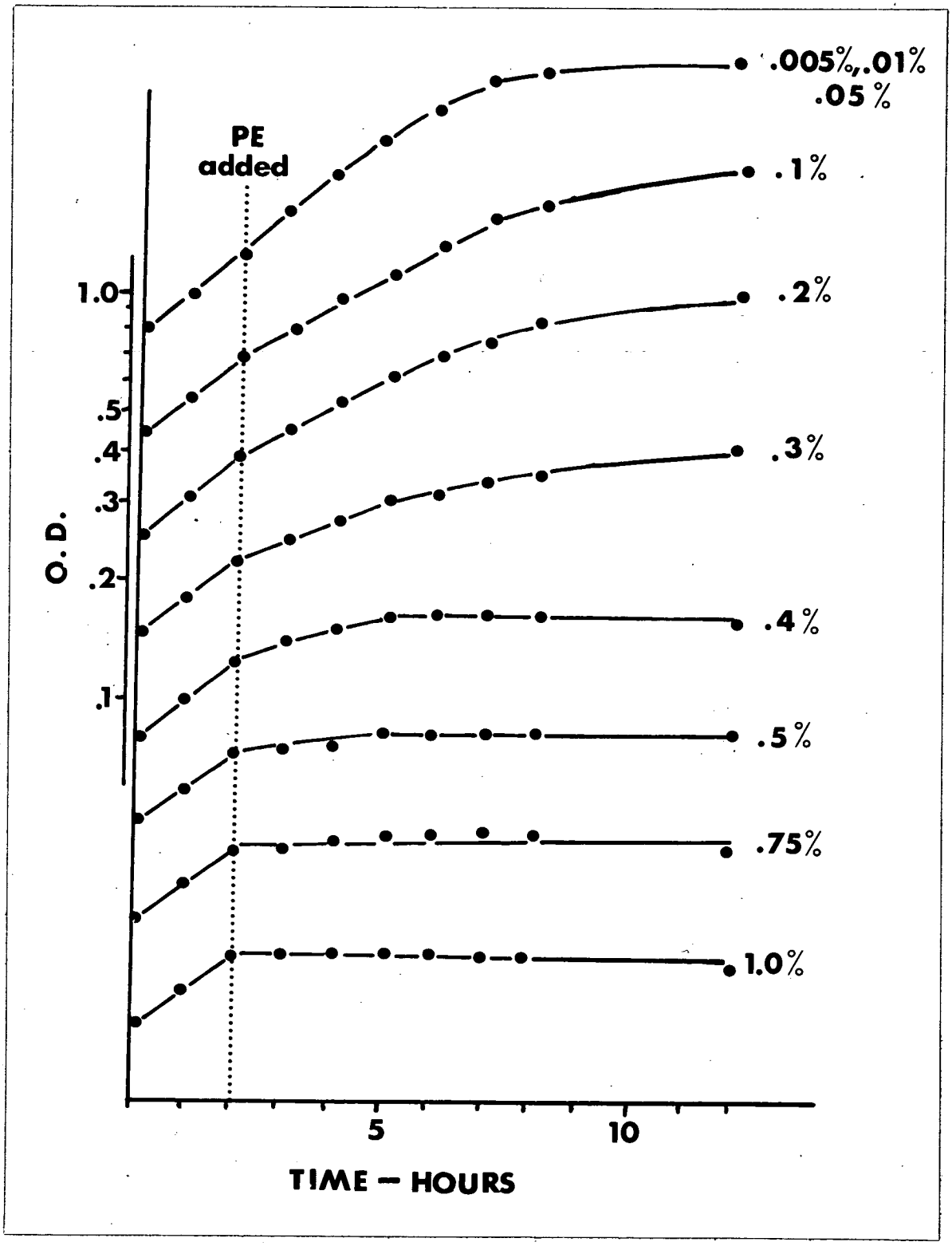
Chapter 9

The Effect of 2-Phenyl Ethanol on the DNA Synthesis Cycle of Schizosaccharomyces pombe

Results and Discussion

In previous reports describing the action of 2-phenyl ethanol (PE) there was considerable variation in the concentration of PE necessary to produce an inhibition of DNA synthesis, depending on the type of cell that was under examination (Bruchovsky and Till, 1967; Leach et al., 1963; Rosencranz et al., 1965; Gabriel, 1965), and the type of culture medium which was being used (Trieck, 1966). Several test cultures of S. pombe were set up, to which were added varying concentrations of PE. Growth of these cultures was monitored by optical density measurements, with occasional observations on cells under the microscope. Figure 31 shows the results of one of these experiments. Little or no effect was observed on the growth of cells by the presence of PE up to a concentration of 0.1%. At this concentration the growth rate slowed down slightly, though there was no apparent effect on the size of cells in these cultures. At 0.2% the growth rate was reduced still further and the final yield also reduced. This effect became even more pronounced at 0.3% and at concentrations of 0.4% or more virtually all growth was stopped upon addition of the inhibitor.

Figure 31. The effect of various concentrations of 2-phenyl ethanol on the growth of S. pombe in EMM 2 at 32°C. Growth is expressed as increase in optical density at 595 mμ.


.005%,.01%
.05%
PE
added
.1%
1.0
.2%
.5
.4
.3%
.3
O. D.
.2
.4%
.1
.5%
.75%
1.0%
5
10
TIME — HOURS

For the experimental dissociation of the DNA synthesis cycle from the rest of the cell cycle a concentration of inhibitor that would effectively inhibit DNA synthesis, but which would have little effect on the other macromolecular syntheses, was desirable. The effects of 0,2% and 0.3% PE on DNA synthesis were therefore investigated. The results of these experiments are shown in figures 32, 33, 34 and 35. Cultures of S. pombe were grown up in EMM 2 from a small inoculum, and sampling was started when the cell concentration had reached 2 x 10^6 cells/ml. The growth of these cultures was followed in terms of cell number and DNA content. After a short period of growth and sampling, to establish the normal rate of increase of both parameters, PE was added to the required concentration and the culture was thoroughly shaken.

In 0.3% PE (figures 32 and 33) there was an immediate inhibition of all DNA synthesis, but there was an approximate doubling in the cell number. At the time of addition of PE the mean amount of DNA/cell was 0.0376 µµg. DNA/cell (s.e. of mean = 0.0005), but this dropped steadily after the addition of PE to reach a value of 0.0201 µµg. DNA/cell at the end of the experiment. This concentration of PE completely inhibited DNA synthesis, but allowed growth and division to continue for a limited period. The cells did not, however, become unusually long, as they do in the presence of Mitomycin C (Herring, pers. commun.) so the presence of PE was limiting to some extent the

Figure 32. Changes in cell concentration (open circles) and DNA per sample (solid circles) following addition of 0.3% PE to an EMM 2 culture at 32°C.

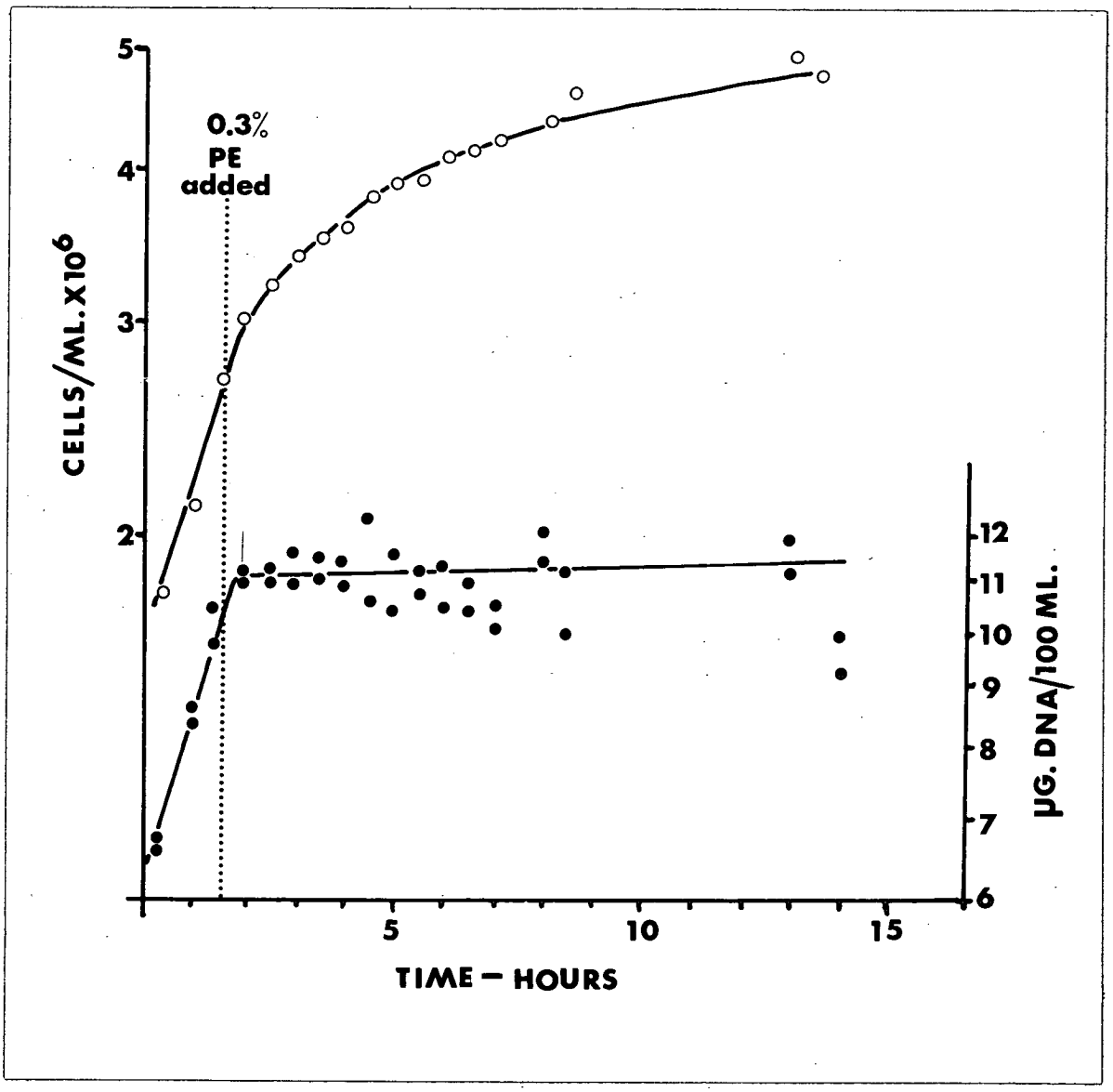

0.3%
PE
added
CELLS/ML. X10^6
5
4
3
2
12
11
10
9
8
7
6
µG. DNA/100 ML.
5
10
15
TIME — HOURS

Figure 33. Changes in the amount of DNA per cell following addition of PE to 0.3% to an EMM 2 culture at 32°C.

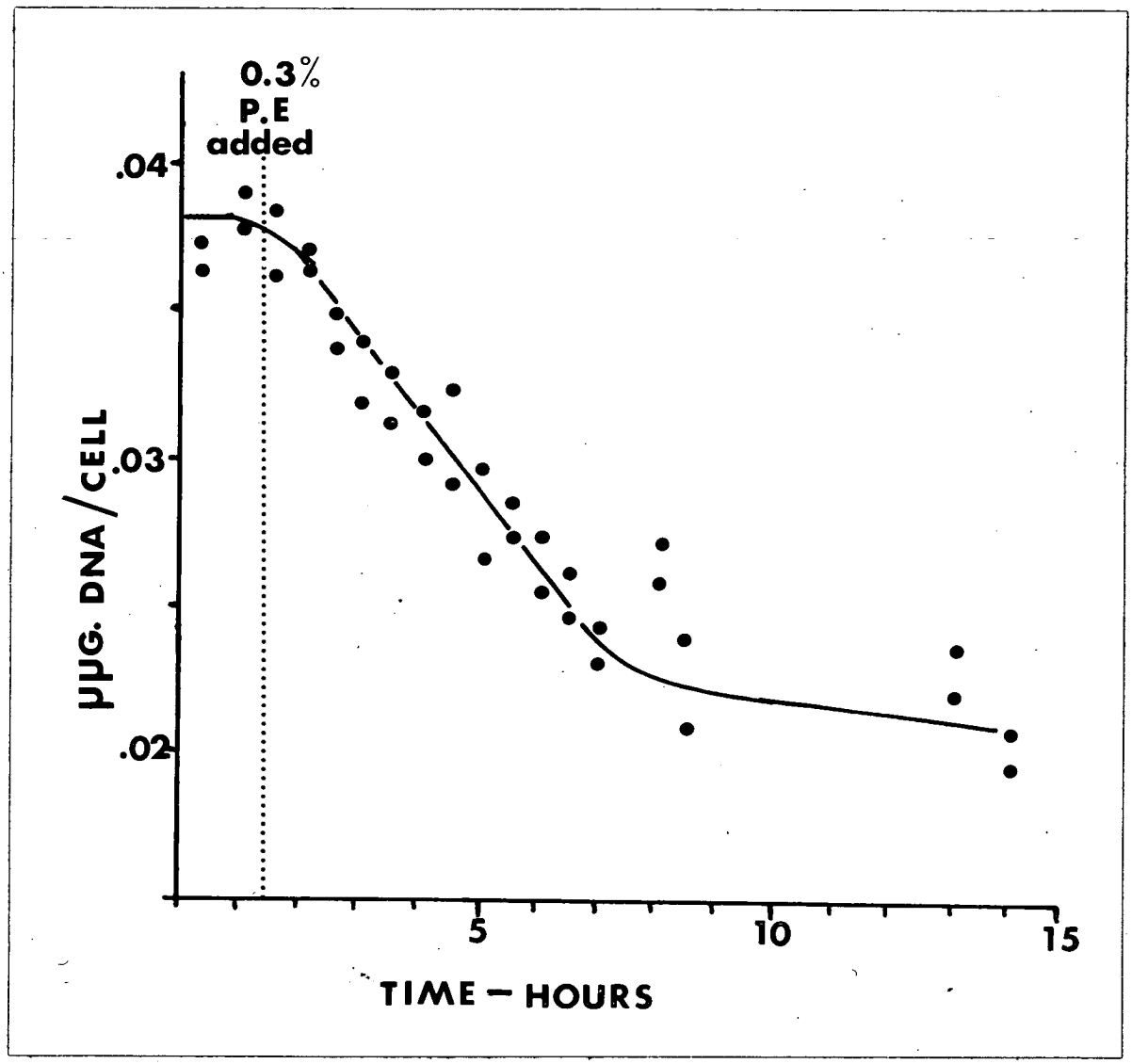

0.3%
P.E
added
.04
.03
.02
μμG. DNA/CELL
5
10
15
TIME – HOURS

Figure 34. Changes in cell concentration (open circles) and DNA per sample (solid circles) after addition of PE to 0,2% to an EMM 2 culture at 32°C.

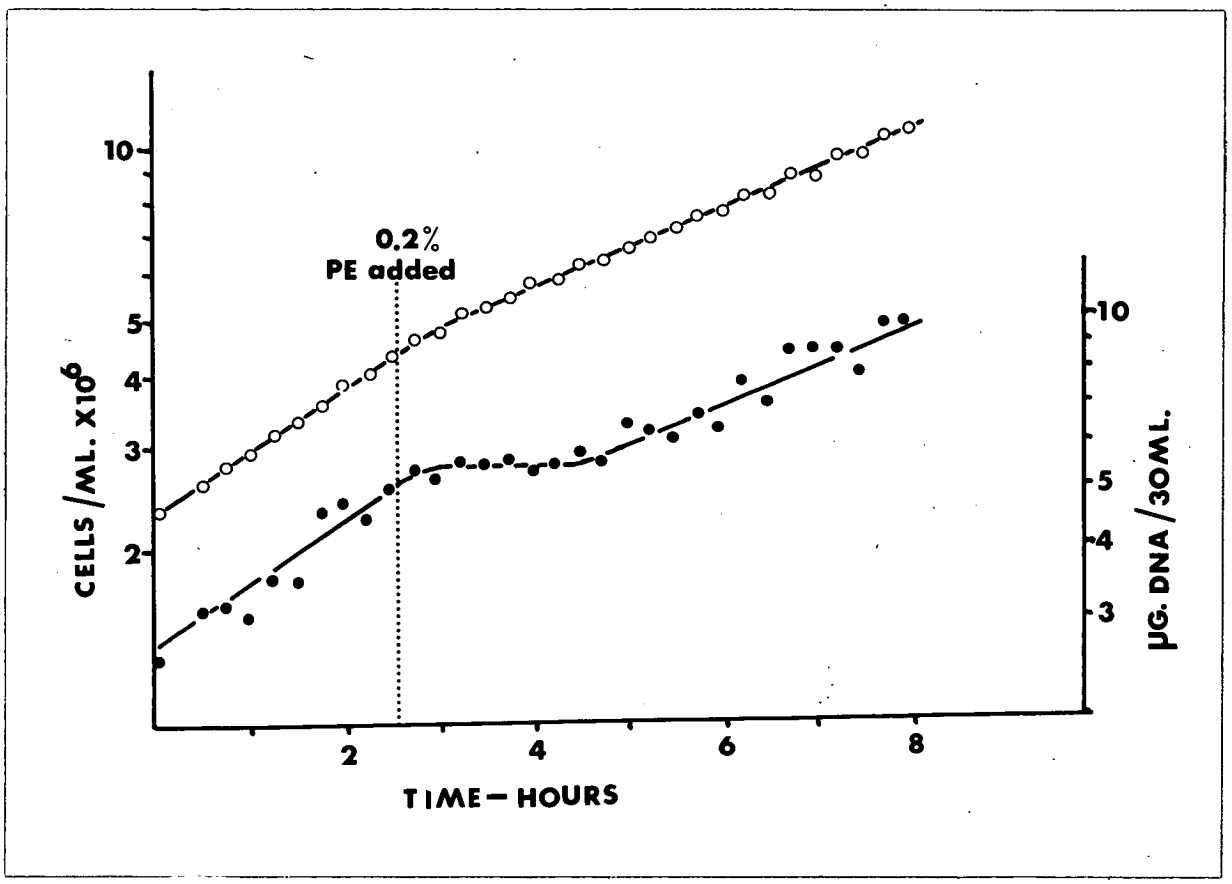
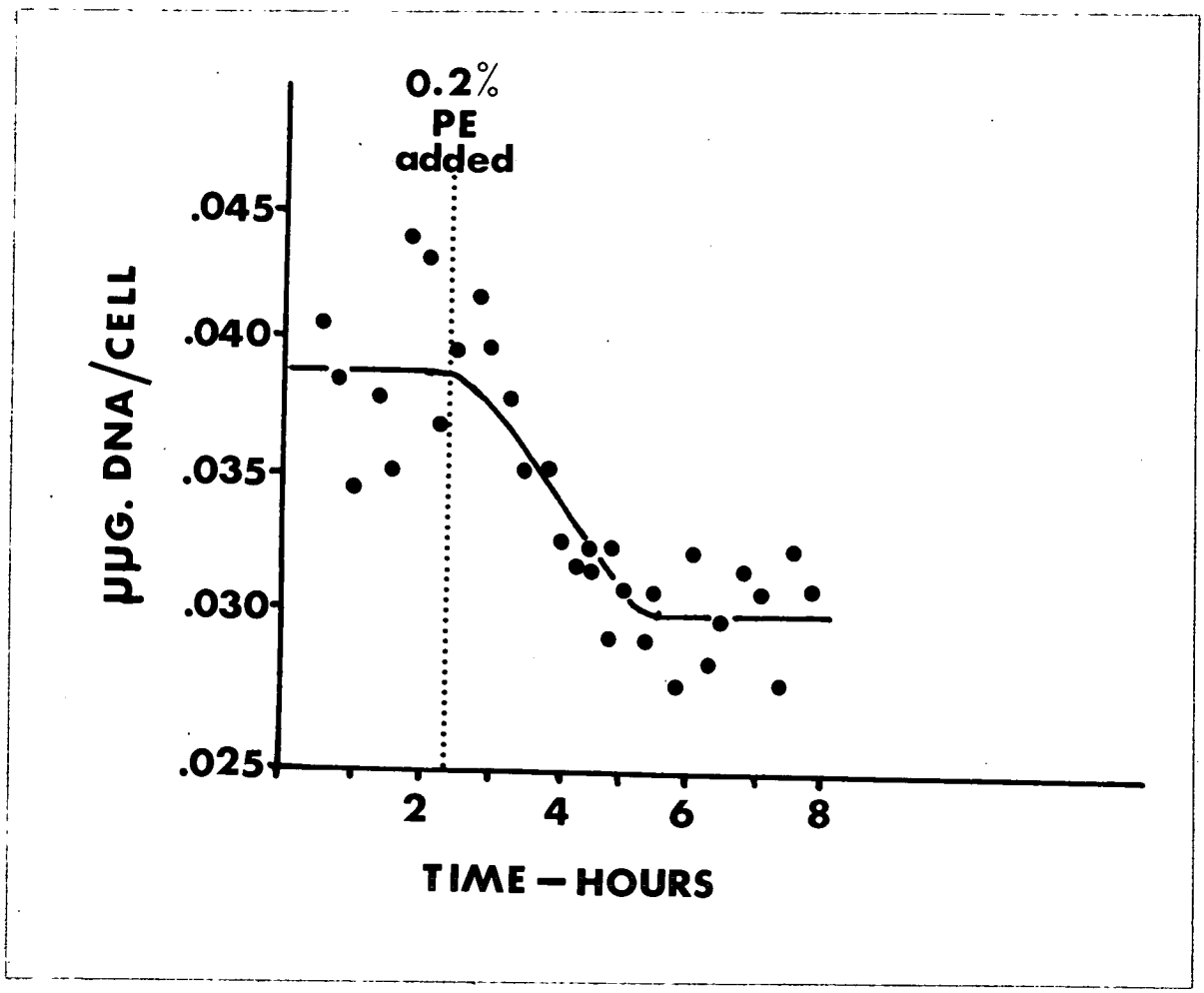

10
5
4
3
2
0.2%
PE added
CELLS /ML. X10^6
10
5
4
3
µG. DNA /30ML.
2
4
6
8
TIME — HOURS

Figure 35. Changes in the amount of DNA per cell following addition of PE to 0.2% to an EMM 2 culture at 32°C.


0.2%
PE
added
.045
.040
.035
.030
.025
μμG. DNA/CELL
2
4
6
8
TIME — HOURS

amount of growth possible in the absence of DNA synthesis. One interesting point of this experiment was the fact that, in the absence of any detectable DNA synthesis, the culture doubled in cell number. There is a short or absent G1 phase, so the majority of the cells were therefore inhibited in G2, with a few cells in the two 1c or two 2c nuclei stage. Theoretically, if the S phase could be displaced in the cell cycle and fission take place in the absence of the previous DNA replication, there should be a little more than a doubling of cell number, and the mean DNA content of cells should drop to the 1c value. This condition was not quite reached in these experiments, and even after 24 hours following the addition of the inhibitor the mean amount of DNA per cell did not decrease any further.

The effect of 0.2% PE was rather different from that of 0.3% PE and showed a peculiar phenomenon of this yeast cell. Figure 34 shows the results of one of these experiments. The experimental procedure was the same as for 0.3% PE described above. After the addition of PE the rate of increase in cell number decreased slowly and stabilised at a generation time of about 4 hours 15 minutes. (Growth could be maintained into stationary phase at this growth rate, and cultures maintained indefinitely by subculturing in EMM 2 plus 0.2% PE.) Shortly after the addition of PE at this concentration the increase in the amount of DNA in the culture stopped, and remained constant

for about two hours. After this, synthesis of DNA recommenced in the culture with a rate of increase equal to that of cell number. When this data is plotted as DNA/cell (figure 35), it can be seen that the overall mean DNA content of cells before the addition of PE was 0.0389 μμg. DNA/cell (s.e. of mean = 0.0001), and that this dropped to a value of 0.0300 μμg. DNA/cell (s.e. of mean = 0.0005) once DNA synthesis started up again after the temporary inhibition.

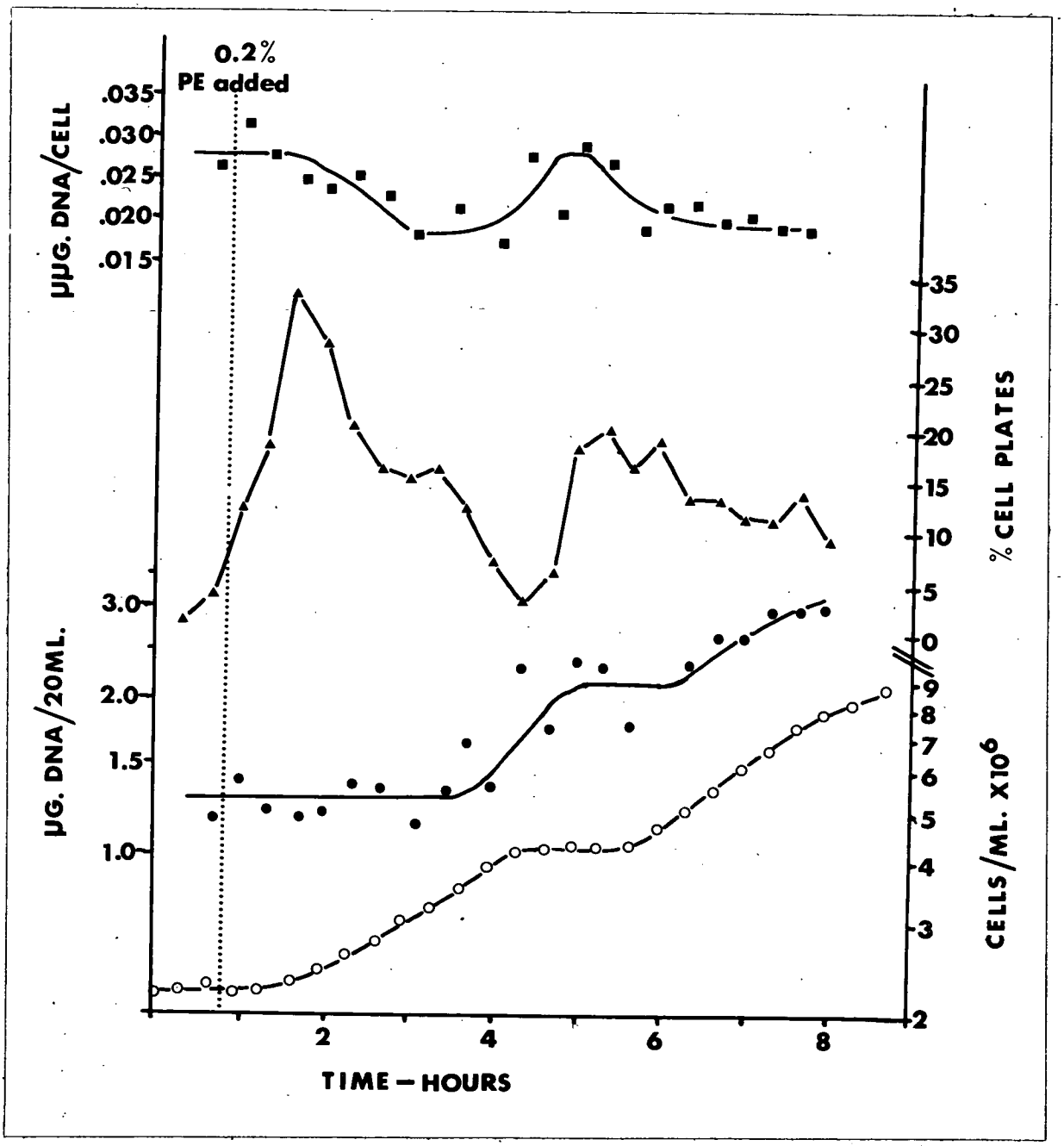
The possibility existed that the cells in some way reduced the effective concentration of PE in the medium, and hence overcame the inhibitory effect. PE has a very characteristic absorption pattern in the ultraviolet spectrum, and during the growth of the culture samples were removed, the cells spun down by centrifugation, and the absorption spectrum measured on a Unicam SP 800 recording spectrophotometer. At all times during the growth of the culture the absorption spectrum and the peak of absorption at 256 mμ remained the same, suggesting that the inhibition and subsequent recovery of the cells was due to some other factor than a change in the concentration of the inhibitor in the medium.

There are two possible explanations of the change in the mean DNA content of 0.2% PE treated cells. Firstly, the presence of this substance could have been lengthening the proportion of the cell cycle spent in S phase, without affecting the time of initiation of S. This would have had

the effect of pushing back the point at which half the DNA was synthesised in the cell cycle. If this was happening one would not have expected to have got a plateau in the DNA curve after the addition of PE, rather a much slower rate of increase of DNA which would have gradually increased to equal the rate of increase in cell numbers after one generation time (4¼ hours). The second possibility is that the PE was inserting a G1 phase which was approximately equal to the observed length of DNA synthesis inhibition. In this situation one would have expected a complete cessation of DNA synthesis after the addition of PE, except for those cells in the S phase at the time of addition which would have completed that round of replication. At the end of the 'new' G1 synthesis of DNA would have taken place at the rate per cell present in normal cells. The S phase would, therefore, have been in mid cycle, with the effect that, in a random log. phase culture, the mean amount of DNA per cell would have been less than in the normal culture. It is not possible to distinguish between these on the basis of the results so far, due to the scatter in the estimation of the DNA content of the culture. However, such a distinction could be made on the basis of the results from synchronous cultures.

There were two types of synchronous culture experiment. The first, of which the results of one are shown in figure 36, involved the making of a normal EMM 2 synchronous culture with

Figure 36. The effect of adding PE at 0.2% to an EMM 2 synchronous culture of S. pombe. The effect was followed in terms of cell concentration (open circles), DNA per sample (solid circles), DNA per cell (squares) and cell plate index (triangles).


0.2%
PE added
μμG. DNA/CELL
.035
.030
.025
.020
.015
μG. DNA/20ML.
3.0
2.0
1.5
1.0
% CELL PLATES
35
30
25
20
15
10
5
0
9
8
7
6
5
4
3
2
CELLS/ML. X10⁶
2
4
6
8
TIME — HOURS

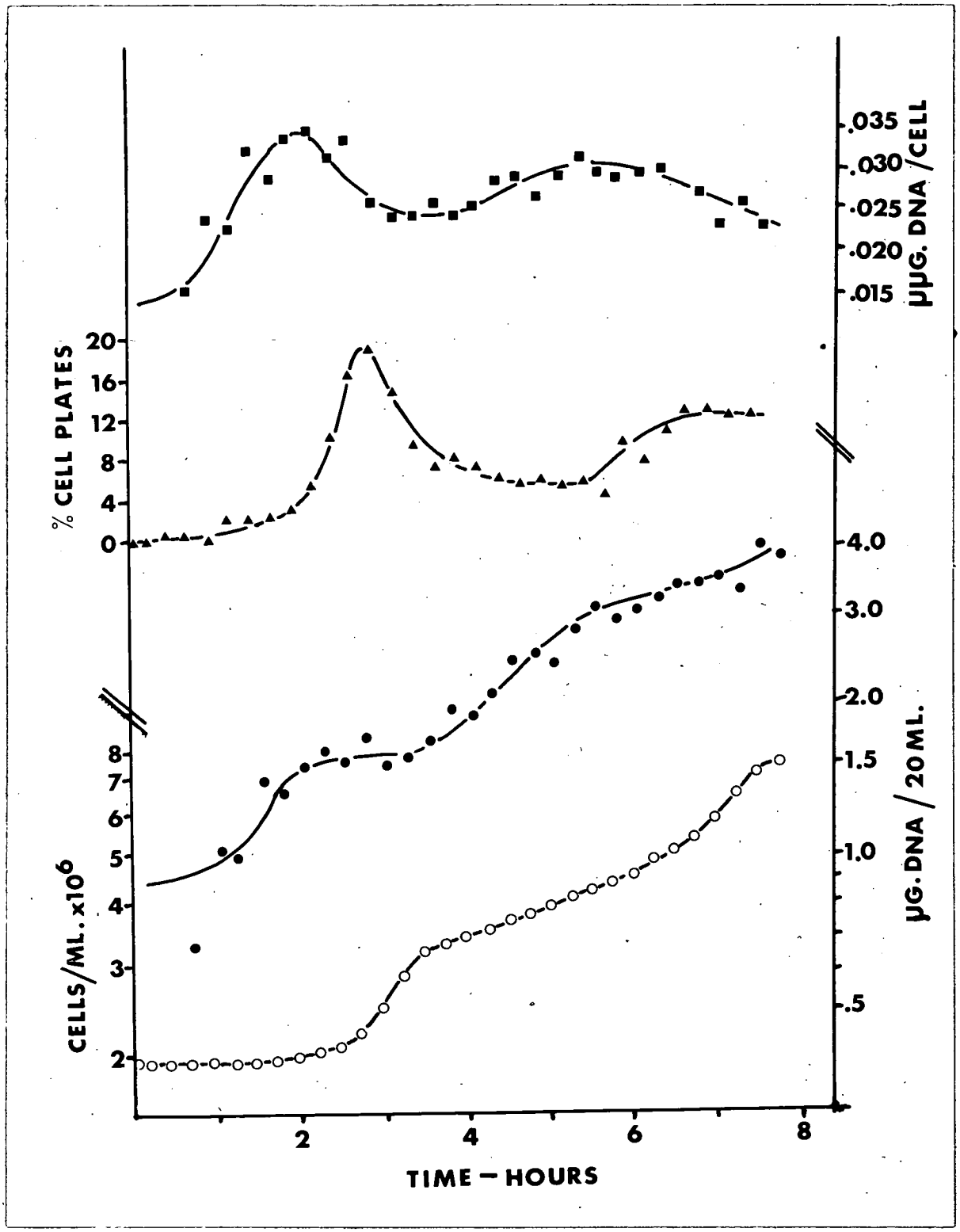
the addition of PE after 45 minutes of synchronous growth. The addition of PE to these cultures destroyed to a large extent the first synchronous divisions and there was little evidence of any synchrony in the subsequent divisions. This was probably due to the fact that PE increased the variability of the size at which cells divided, as a high proportion of cells divided during the first wave of divisions with acentric cell plates. Despite this effect some degree of synchrony was observed in the first division and this could be used to position the S phase in the cell cycle.

Figure 36 shows the increase in cell number and DNA per 20 ml. sample in a 0.2% PE synchronous culture. A comparison between this result and those in the absence of the inhibitor (chapter 3) shows that there is a marked difference between the two. The mid point of the rise in the amount of DNA per sample occurred about 1½ hours after the mid point of the rise in cell numbers in the experiment shown in figure 36. Similar results were found in repeats of this experiment. When the results are plotted in terms of DNA per cell it can be seen that, upon inoculation, the cells had a mean DNA content characteristic of 2c cells. In the normal synchronous culture this value was maintained throughout the growth of the culture, except for a possible increase at the time of cell division. In PE cultures, however, because the synthesis of DNA was delayed, and the cells divided before the S period, the mean

amount of DNA per cell dropped, at the time of division, towards the 1c value. It never reached this value, probably because of the lack of good synchrony at this stage, but these results clearly show that the addition of PE to cultures of S. pombe in EMM 2 pushed back the S period in the cell cycle.

The second type of synchronous culture differed from the above in as much as the cells, which were grown up prior to separation on the gradient, had been cultured in the presence of 0.2% PE for about ten cell generations. Also, harvested cells were separated on a gradient which contained 0.2% PE. The cells had had several generations in which to settle down to the presence of PE in the medium. The results of one of these experiments are shown in figure 37. As in the previous experiments the degree of synchrony was not good, but sufficiently so to establish the position of the S phase in the cell cycle. It can be seen that both the cell numbers and the amount of DNA per sample increased discontinuously, and that the mid point of the increase in the DNA curve was about two hours before the mid point of the rise in cell numbers. In this experiment the cells that came off the top cell layer in the gradient to make the synchronous culture were in G1, because the mean amount of DNA per cell was close to the 1c value. Shortly after inoculation these cells entered S phase and the mean DNA content of cells increased to the 2c value. Once the first wave of divisions began this was reduced again

Figure 37. Cell concentration (open circles), DNA per sample (solid circles), DNA per cell (squares) and cell plate index (triangles) in an EMM 2 plus 0.2% PE synchronous culture of S. pombe. The cells had been grown on EMM 2 plus 0.2% PE for several generations prior to synchronisation.


% CELL PLATES
20
16
12
8
4
0
μμG. DNA /CELL
.035
.030
.025
.020
.015
CELLS/ML. x10⁶
8
7
6
5
4
3
2
μG. DNA / 20 ML.
4.0
3.0
2.0
1.5
1.0
.5
2
4
6
8
TIME — HOURS

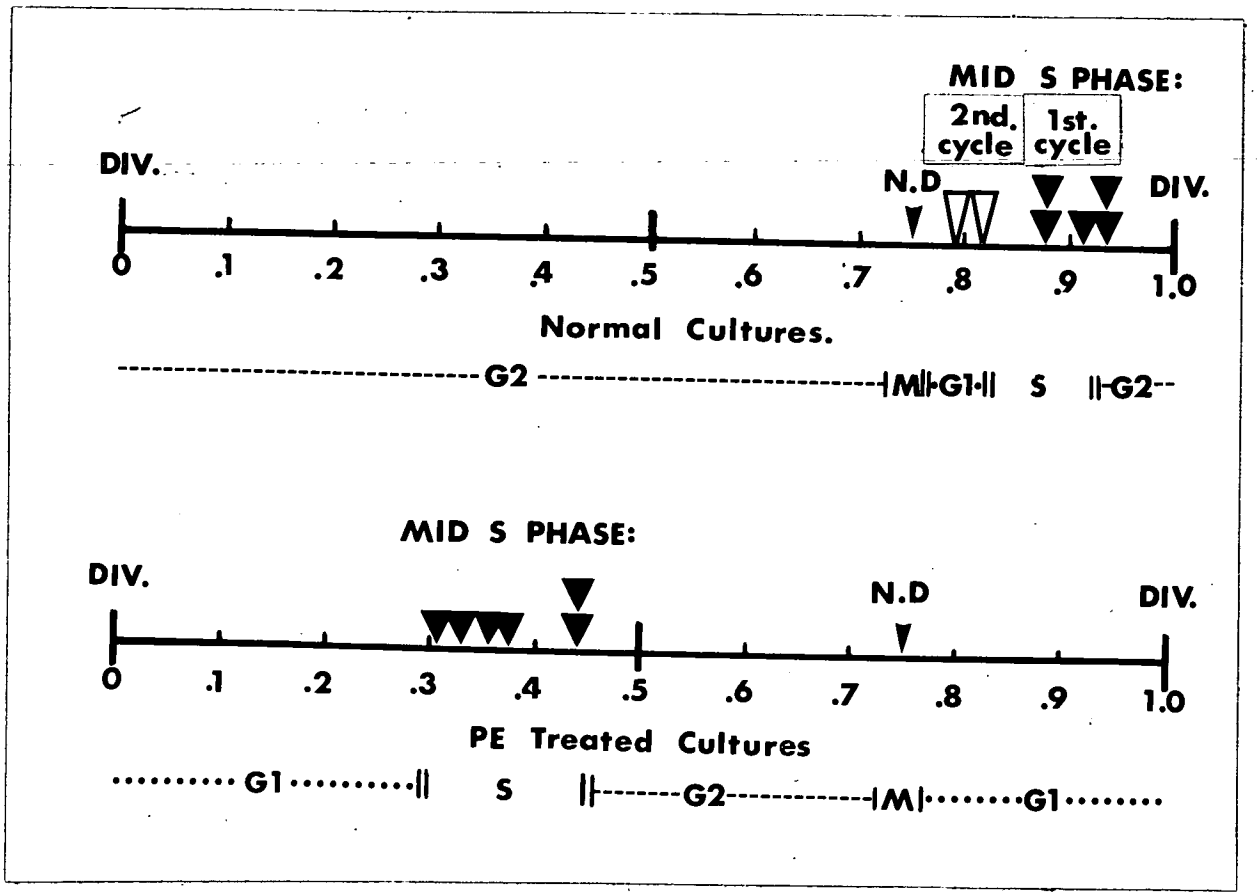
as single G1 cells were formed. As in the previous experiment the 1c value was never reached, but the fluctuations in the mean amount of DNA per cell, between the 1 and 2c values again showed that, in the presence of this inhibitor at this concentration, the DNA synthesis cycle had a large G1 phase.

It is therefore possible to distinguish between the two possible explanations of the effects observed after the addition of 0.2% PE to random cultures. There are two features of the synchronous cultures which rule out the possibility of a greatly extended S phase. These are the presence of 1c cells and hence a G1 phase, which has already been discussed, and secondly the observation that the shape of the DNA curve was very similar in both types of experiments to that of the increase in cell number. This suggests that the S phase was of the same order of time as the division process, which was also the case in normal cultures. As such the S phases in both normal and PE cultures were of comparable lengths, and there is no indication that there was any effect on the S phase as such in the treated cultures.

Another significant point to emerge from the PE cultures is the fact that the G2 phase was of similar absolute length in both normal and PE treated cultures. In normal cultures the cells grew with a generation time of 2 hours 20 minutes and the time between the end of nuclear division to the end of the S phase was about 30 minutes. G2 in normal cultures therefore

lasts for approximately 1 hour 50 minutes. The normal DNA synthesis cycle has been reproduced in figure 38, together with the position of the S phase in the PE treated synchronous cultures, derived from the relative positions of the mid points of increase of the DNA per sample and cell per ml. curves. The time between these two has been plotted as a fraction of the total generation time, and figure 38 includes the results from six PE treated cultures. From this it can be calculated that the length of the G2 phase in EMM 2 plus 0.2% PE is about 1 hour 40 minutes, assuming the interval occupied by the S and M phases to be the same as in normal cultures, out of a total generation time of 4 hours 15 minutes. Thus both the S and G2 phases are of the same order of absolute lengths in both the normal and experimental situations, the major difference in the growth rates between the two culture conditions being the result of an extended G1 in the presence of 0.2% PE.

Figure 38. Cell cycle maps showing the difference in the position of the S phase in S. pombe when grown in the presence or absence of 0.2% PE in EMM 2.


MID S PHASE:
2nd. cycle
1st. cycle
DIV.
N.D
DIV.
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
Normal Cultures.
G2
M
G1
S
G2
MID S PHASE:
DIV.
N.D
DIV.
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
PE Treated Cultures
G1
S
G2
M
G1

Chapter 10

General Discussion

The results described in the first part of this thesis show that S. pombe, when grown at 32°C on EMM, has a rather unusual DNA synthesis cycle. This cycle consists of a short, or possibly absent, G1 phase, a short S phase and a long G2 phase occupying the majority of the cell cycle. The rest of the cycle is occupied by nuclear division, and the exact length of this process will determine the absolute length of the G2. For example, in S. cerevisiae Williamson (1966) has shown that chromosome separation occurs some time before the nucleus undergoes visible fission. In higher cells the process of chromosome separation and nuclear fission are both considered parts of mitosis, or the M phase. If the same criteria for the M phase are applied to this yeast, the distinction between G2 and M becomes difficult at present, without further information about the structure of the nucleus during the cell cycle. It is probable, though, that some form of chromosome separation occurs prior to the separation of the daughter nuclei, so the M phase is probably longer, and G2 shorter, than the fission and migration of nuclei suggests.

This cell cycle is peculiar in many respects when it is compared to the situation in the majority of other cell types that have been examined (see discussion in chapter 1). In

these all the stages occupy variable, but significant, portions of the cell cycle, so the main differences between S. pombe and these cells is the absence of a G1, detectable by the techniques used in this study, and a very short S phase. Unusual though these features are they are not unique amongst animal, plant or bacterial cells.

During the early development of amphibian embryos the cell cycle is very short, of the order of 15 minutes, and there is no detectable G1 phase and only a very short G2 phase (Graham and Morgan, 1966). Therefore, during the first four hours after fertilisation the S phase occupies the majority of interphase, but is relatively short compared to that observed during the later stages of development. By the time the embryo has reached the late blastula stage a G1 and a substantial G2 can be detected, and there is also an absolute increase in the length of the S phase. From the late blastula to the tail bud stage the lengths of all three phases increase, but M stays relatively constant. Once the embryo has reached the gastrula stage the increases in G1, S and G2 are such that the portion that each occupies in the cell cycle remains constant (Graham and Morgan, 1966).

A similar process occurs in the cells of the root of Zea mais (Clowes, 1965). The cell cycle of the cap initial cells of the root meristem is shorter than elsewhere in the plant, and it lacks a measurable G1. As the generation time

increases, due to development into the quiescent zone or to differentiation into stele cells, a G1 phase is inserted which almost accounts for the increase in the generation time. Thus in the rapidly dividing tissue there is no G1, but as the generation time increases so does the proportion of the cycle occupied by G1.

The early divisions of sea urchin eggs show great similarities to those of the amphibian embryos. Hindegardner et al. (1964) showed that, at 15°C, the first division cycles last for 2 hours, of which 13 minutes are occupied by the S phase, the remainder being G2 and M. DNA synthesis starts after the first quarter of telophase and ends at the beginning of interphase, there being no G1. No information is available about the pattern of DNA synthesis during later stages of sea urchin development, but it seems probable that, as the rate of cell division decreases, the proportion of the cell cycle occupied by G1 and/or G2 would increase, in much the same way as it does in the previous two examples.

Another embryonic cell system that has been studied, and which shows essentially the same characteristics, is the neuroblast cells of grasshopper embryos (Gaulden, 1956). In these cells the cell cycle lasts for four hours and the S phase starts at telophase and continues throughout interphase to early prophase. There is therefore no G1 or G2 in these cells. The absence of a G1 is also shown by the early work of Dalcq and

Pasteels (1955) in their studies on the DNA content of rat and mouse embryonic cells during the first few divisions following fertilisation. In these they showed that the DNA content of a nucleus begins to increase at the end of telophase.

The absence of a G1 phase is not restricted to these rather special developing cell systems. It has also been shown in a cultured strain of Chinese hamster lung cells (Robbins and Scharff, 1967), which had been synchronised by the selection of metaphase cells. It is possible that the synchronisation technique had an effect on the cells, but it is significant that the generation time of these cells was less than is normal for mammalian cells. Another example of a cell that has no G1 phase is Amoeba proteus, but in this case the rate of cell division is not rapid. The S phase lasts for 6 to 8 hours and the G2 phase for about 30 hours (Prescott and Goldstein, 1967). In T. pyriformis, grown at a generation time of 3.7 hours on enriched synthetic medium, the micronuclear S phase begins at telophase and continues during the process of cytokinesis, occupying 10% of the total cell cycle (Flickinger, 1967). In the same organism the macronuclear S phase is in mid cycle, so whatever factors, or levels of them, are controlling the initiation of micronuclear DNA synthesis, they are not sufficient for the initiation of macronuclear DNA synthesis. Whether or not a G1 phase is inserted into the micronuclear cycle when the organism is grown at slower growth

rates is not known, but as environmental factors can affect the position of the macronuclear S period (Cameron and Nachtwey, 1967), there are reasonable grounds for expecting the micronucleus to behave likewise. If such a phenomenon was observed it would be very similar to the situation that arises during phosphate starved growth in S. pombe. That is to say that the S phase would be shifted to a point after cytokinesis.

Another example of a cell cycle that has no G1 phase is the slime mould Physarum polycephalum. Nygaard et al. (1960) studied the incorporation of labelled orotic acid into DNA at various intervals following synchronous mitosis in this organism. They showed that the S phase commenced immediately after mitosis and lasted for between 1 and 2 hours out of a total cycle time of between 12 and 20 hours. In this organism, therefore, the majority of the cell cycle is taken up with G2.

All the preceding examples show similarities to various aspects of the DNA synthesis cycle of S. pombe. The first, and most obvious, is the similar distribution in the lengths of the various phases of the cell cycle, and in particular the lack of any detectable G1 phase. But the similarities go further than this. In some of the systems described above G1 phases are inserted in the DNA synthesis cycle as a normal consequence of development and differentiation of the cells. As cultures of S. pombe begin to enter stationary phase similar changes occur in the DNA synthesis cycle; the growth rate

falls off under conditions of limiting phosphate and cells begin to possess a G1 phase. When cultures are maintained under conditions that simulate the conditions present in the early stationary phase culture medium the changed pattern of DNA synthesis is observed and repeated for several cell generations. The conditions that cause a slowing down in the rate of cell division in the embryos or the root meristem are not known, but clearly some 'environmental' factor must be involved, and, though the factors are no doubt different in each case, they produce very similar effects.

The decrease in the rate of cell division due to alterations in some environmental factors is not the sole requirement for the existence, or extension, to the G1 phase. In Amoeba proteus, for example, the generation time is of the order of 30 hours, which is not rapid by any standard, and the total cell cycle in Physarum polycephalum is between 12 and 20 hours, which is similar to the duration of the cell cycle of, for example, mammalian cells, which generally show a distinct G1 (Cleaver, 1967). For cells that normally possess a significant G1 phase it is this phase that shows the greatest variability, and in general it is this phase that accounts for the majority of the increases in generation times. This is so both between and within cell types (Cleaver, 1967; Terasima and Tolmach, 1963; Sisken and Morasca, 1965), growing under constant conditions, or between the same cell types growing under

different conditions (Defendi and Manson, 1963; Sisken et al., 1965; Wimber, 1966; Cameron and Nachtwey, 1967; Helmstetter and Cooper, 1968). There are differences in the responses of cells to changes in the growth rates due to temperature shifts depending on whether the cells are in the process of adjusting to the new temperature or whether they are in a steady state at the new temperature (Rao and Engelberg, 1966).

The effect of temperature, pH or nutritional changes in the culture conditions must ultimately be to change the intracellular environment for, amongst other things, DNA synthesis. Such changes will only have an effect on the pattern of the DNA cycle when the conditions necessary for DNA synthesis are limiting. In this respect it is interesting to note that in the sea urchin egg, in which there are large amounts of macromolecular precursors, temperature change has no preferential effect on any of the DNA synthesis phases (Hindegardner et al., 1964). In T. pyriformis, when it is grown on an enriched medium, changes in the culture temperature have no preferential effect on any of the DNA synthesis phases (Cleffman, 1967), but when the culture medium is less enriched a disproportionate extension in the G1 can be observed (Cameron and Nachtwey, 1967). These could be interpreted as showing that unless the environmental changes are sufficient to make a factor limiting, or decrease the level of it in the cell, which is necessary for the initiation of DNA replication, no effect will be observed

on the G1 or S phases of the cell cycle. In much the same way changes in the conditions necessary for the preparation and maintenance of G2 or M could bring about a redistribution of the proportion of the cell cycle spent in each of the phases (e.g. Rao and Engelberg, 1966). The lack of an extension to the G1 phase in S. pombe, when the generation time was increased by lowering the temperature or changing the carbon source, is therefore possibly an indication that neither of these alterations create limiting levels in the factors involved in the initiation and maintenance of the S phase.

The conditions that do introduce a G1 phase in S. pombe, limiting phosphate and the presence of PE, have more specific implications in the synthesis of DNA than the factors that have been mentioned in the previous paragraph, and it is easier to imagine how they could be influencing the change in the DNA synthesis cycle. The presence of phosphate is necessary for the production of deoxyribonucleotide triphosphates, and in this connection it is interesting to note that the first sign of a change in the rate of synthesis of DNA in random EMM 1 cultures coincides with the time at which the level of phosphate in the pool begins to drop.

Phenyl ethanol has been shown to inhibit the initiation of the S phase in bacteria (Lark, K., and Lark, C., 1966; Trieck, 1966), possibly by interfering in some way with the association of DNA with the initiator site. Alternatively, if the primary

site of action is mRNA (Rosencranz et al., 1965; Slepecky and Celkis, 1964) then the inhibition could be through the lack of synthesis of the initiator site. Further evidence for the involvement of PE in the alteration of some DNA/protein interaction comes from the work of Mendelsohn and Frazer (1965). In in vitro studies they showed that PE had little effect on DNA strand separation or on its viscosity, but that it had a marked effect on the size of intact phage heads. In the presence of the inhibitor the protein coat surrounding the phage DNA was found to shrink, though such an effect was not found in the absence of DNA. The shrinkage appeared to be DNA dependent.

Within the framework of DNA/protein interactions there are three possible sites at which the PE could be acting. The first is that it may be altering the relationship between the DNA molecules and a hypothetical initiator protein, somewhat analogous to that of the bacteria (Jacob et al., 1963). Perhaps PE enhances the complexing of the DNA to the initiator site, making it more difficult for one of the mother strands to separate and attach to a new initiator site, or it may act by competing with the DNA for attachment sites on the protein, or vice versa. If the effect was the result of competition it should be possible to distinguish on the basis of more information, for with each halving of the concentration of PE in the medium the length of the G1 should be halved. Equally for

each doubling in the concentration the G1 should double, which was not the case when the concentration of PE was raised from 0.2 - 0.3%. However, at the higher concentrations of the inhibitor other effects could be operating to mask the effect on DNA synthesis.

Alternatively it could be having an effect on the association of DNA polymerase with DNA such that there is a lag, the observed G1, before DNA synthesis can commence. In this respect it is interesting that there is an analogous lag in the *in vitro* synthesis of polynucleotides by partially purified polynucleotide phosphorylase (Grunberg-Manago, 1963). Grunberg-Manago suggests that there is a specific threshold concentration of oligonucleotide primer required before synthesis of the polymer can begin. In *S. pombe* the G1 may represent the time that is necessary for the synthesis of some intermediate or co-factor which is required for the polymerase mediated DNA synthesis. In this case one would expect a gradual increase in the rate of DNA synthesis as more intermediate is made, unless an all or none effect is operating due to a threshold concentration phenomenon. The data are not sufficiently good to tell whether the start of the S phase is gradual or rapid in the presence of PE.

The third possibility is that PE is affecting the association of histones and proteins with the DNA, so that different physical conditions are required before the two can dissociate

prior to synthesis. The observation that, in the presence of PE, phage head shrinkage occurs suggests that there is a tighter association between the two, which, in the continued presence of the inhibitor, might require more extreme conditions for separation to occur. That separation of histones and DNA must occur prior to synthesis of DNA is suggested by the inhibitory effect of the former in in vitro DNA synthesising systems (Bitten and Hnilica, 1964). In this case the G1 reflects the time it takes for the nuclear environment to change in such a way that synthesis can occur. The dissociation between the proteins and DNA is likely to be dependent on many factors, but once the correct environment is present it could be as rapid as in normal cells, resulting in a distinct change from G1 to S. Once the S phase has started it is likely to be of the same order of time as that in normal cells because the conditions necessary for the dissociation of proteins from DNA would be throughout the nucleus.

It has been suggested that the primary site of PE inhibition is messenger RNA, but it is unlikely to be the case for S. pombe grown in 0.2% PE, because under these conditions the net synthesis of proteins is restricted to G2 (Stebbing, pers. commun.). During this period of the cell cycle the amount of protein doubles in much the same way as it does in normal cells. If the primary effect was an inhibition, or a slowing down, in the rate of mRNA synthesis the increase in the

amount of protein would be expected to occur over the whole cell cycle. It may be the case that whatever is causing the delay in the initiation of the S phase is also causing a delay in the initiation of transcription, and that, once the conditions necessary for the initiation of S are met, transcription may also proceed.

Another peculiarity of the S. pombe cell cycle is the extremely short S phase. Although it was not possible to get a precise measurement of the length of time that cells spend in DNA synthesis, the shapes of the DNA curves in the synchronous and lag phase cultures suggest that it is of the same order of time that the cells take to complete fission. This is a fairly quick process and probably does not take longer than 10 minutes, so the length of the S phase must be about the same. It is short compared to the majority of DNA synthetic periods observed in other organisms, for example, 20 minutes in E. coli (Helmstetter and Cooper, 1968) and in the micronucleus of T. pyriformis (Flickinger, 1967), 30 minutes in S. cerevisiae (Williamson, 1965), 80 minutes in the macronucleus of T. pyriformis (Flickinger, 1967), and 6 - 8 hours in Amoeba (Prescott and Goldstein, 1967), plant cells (Clowes, 1965) and many vertebrate cells in vivo and in vitro (Cameron, 1965; Cleaver, 1967). Such a list is of little significance without the details of growth rates and the conditions and temperature of cell culture. Nevertheless it does show that there are large

differences in the absolute lengths of the S phase and that these are, on the whole, of greater length than that observed in S. pombe.

To find examples of cells that have their S phases of similar length to that in S. pombe one has to return again to the sea urchin and amphibian early embryos. In the former the S period lasts for 13 minutes at 15°C (Hindegardner et al., 1964), and in the amphibian early cleavage stages for about 12 minutes (Graham and Morgan, 1966). Both these organisms have a much higher DNA content per cell, 1.8 μμg. in the sea urchin, than is the case in S. pombe and there are theoretical difficulties over this rapid synthesis. These are overcome by the knowledge that synthesis can be initiated at many different sites along individual chromosomes of higher cells (Evans, 1964; Taylor, 1963).

Freese and Freese (1963) in an analysis of the rate of strand separation in DNA in vitro suggest that the minimum time for the synthesis of DNA in vivo will depend upon the rate at which the parent molecule could uncoil and split into two single strands. This process is in turn dependent on the size of the molecule to be replicated, such that the rate at which separation can take place is inversely proportional to the square of the molecular weight of the replicating molecule. If the relationship that relates these two properties (equation 5 in Freese and Freese) is used to calculate the minimum time

for the S period, it is found that DNA synthesis would take 88 minutes if the DNA in S. pombe was in a single molecule. If, however, one assumes there to be about 18 chromosomes in S. pombe, as there are in S. cerevisiae (Von Borstal, 1966), and each represents a separate replicating unit of DNA, the S phase could be completed in about 20 seconds $\left[\frac{88 \times 60}{18^2}\right]$. This is clearly a value for the minimum time in which the S period could be completed, and in practice some other factor is probably rate limiting, but it does show that the short S phase observed experimentally is theoretically possible. There may be more than one replicating unit on each chromosome, as there are in the chromosomes of higher cells (Evans, 1964; Taylor, 1963), in which case the minimum length for the S phase would become even less.

The S phase also appeared to be constant in length in S. pombe over a considerable range of generation times. The methods used in these studies were not suited to an analysis of the length of the synthetic period, but the results they gave do suggest that there were no large changes in the length of the S phase. This contrasts with the early amphibian development where the increase in the cell cycle is accompanied by a corresponding increase in S, from about 12 minutes during the first divisions to several hours at the gastrula stage and afterwards. What causes this lengthening is not known and it could be due to one of several processes. Perhaps, after the

initial divisions, the levels of one or more of the deoxyribonucleotide triphosphates become limiting such that the rate of synthesis of DNA is dependent on the rate of synthesis of its precursors (Schmidt, 1966). Another possibility is that, after the initial divisions, the amount of polymerase per cell is reduced so that it affects the rate of DNA synthesis. Alternatively there may be some spreading out of the times at which different replication units initiate their synthesis. In differentiated higher cells there is asynchrony of initiation of synthesis along chromosomes (Evans, 1964), and the establishment of this condition would contribute to a lengthening of the total S phase.

Whereas the absence of a G1 phase and the short S phase are unusual properties of cell cycles, the constancy of the G2 phase under different environmental conditions is more universal. The term 'constant' can be used to refer to the length of G2 in either absolute terms or relative terms. In the former, although the experimental conditions may cause an extension in the cell cycle, the length of the G2 would be of the same length as in the normal cycle. However, the relative length of the G2 will have decreased as a result of an increase in one or more of the other phases of the cell cycle (e.g. Sisken et al., 1965; Rao and Engelberg, 1966). Alternatively, the relative length of the G2 phase may not change, due to an absolute increase in its length (e.g. Schaechter et al., 1958).

In practice both these elements arise as a result of environmental changes, but on the whole the absolute increases are less marked than similar changes to the G1 phase.

The environmental changes can be considered in three classes - temperature, nutritional and inhibitor effects. Changes in culture temperature resulted in absolute increases in all the cell cycle phases of S. pombe, with the result that there was no relative change in them. In this respect the DNA synthesis cycle behaves in a similar way to the increase in volume of this organism (Mitchison et al., 1963), in that the patterns of increase over the cell cycle are the same over quite a large range of temperatures. This can be compared to the change in the pattern of dry mass increase when cells are cultured at 17°C (Mitchison et al., 1963), and suggests that the amount of DNA per cell is not closely related to the control of dry mass increase.

The response of S. pombe to changes in the composition of the medium is more complex. When the carbon source was changed to glycerol there were absolute increases in the lengths of the cell cycle phases so that the normal relationship between them was maintained. The response of S. pombe to a culture temperature of 17°C or the presence of glycerol is atypical compared to the other cell systems mentioned in chapter 6. The increases in the generation times due to these treatments was almost entirely a result of an extension to G2,

with no preferential effect on the other phases. With a several fold increase in the generation time some absolute increase in G2 would be expected, but not so much as to account for the total increase in the generation time.

During continuous culture in limiting phosphate a quite different pattern was observed. The mean amount of DNA per nucleus was found to be 0.0151 µµg. DNA, which was not significantly different from the estimated amount in a 1c nucleus. This would suggest that the S phase in these cultures was just before nuclear separation, so that only a small proportion of phosphate starved cells existed in G2. The G2 phase in normal cultures, growing at 32°C, is about two hours, and the generation time of the phosphate starved cells was 24 hours, so a G2 of the same absolute length of that in normal cultures would represent less than 0.1 of the cell cycle. Measurements of the mean DNA content of nuclei would not be sufficiently sensitive to pick up the presence of a small proportion of G2 nuclei, so the results are not inconsistent with the presence of a G2 phase of similar absolute length to that present in normal cultures. Thus, in the phosphate starved continuous cultures the G2 phase probably remained constant in absolute length, but was reduced drastically in relative length.

A more striking demonstration of the constancy, in absolute terms, of G2 is given by the experiments with PE. Although the generation time was increased, and the initiation of the S phase

was considerably delayed in these cultures, the interval between the S phase and the following nuclear division was similar to that in normal cultures.

In the examples cited above either the absolute or the relative length of the G2 remained constant according to the environmental changes that were made. The only time that it might be said that there was both an absolute and relative increase in the length of the G2 was during the lag phase, where it was of the order of 3 - 4 hours, depending on the culture medium and the length of time that cells had been in stationary phase prior to inoculation. The lag phase is, however, a time of unbalanced growth, when the cells are increasing to a size greater than that at any time during logarithmic growth (Johnson, 1968a).

The DNA synthesis cycle of S. pombe is therefore unusual, but some of its features are advantageous for the study of the control of macromolecular syntheses in the cell cycle. The ability to shift the S phase in the cell cycle has advantages when the causal relationships between events are being considered. This ability has been demonstrated in many organisms, but most of the agents that bring about such changes are non-specific for DNA synthesis and it would be difficult to know what would be 'cause' and what would be 'effect'. This can be overcome by the use of inhibitors that specifically attack the DNA synthetic process, but to date such inhibitors

result in the eventual cessation of growth. The effect of PE on S. pombe, therefore, provides an unique system in which the S phase can be specifically displaced in the cell cycle in cell cultures that maintain balanced growth indefinitely. In the presence of 0.2% PE the S phase is moved from about 0.85 in one cycle to about 0.35 in the next, and a change of this magnitude should allow the relationship between the chemical and phenotypic replication of genes to be analysed in more detail. Also, because the inhibitor seems to be affecting the initiation of the S phase, a further examination of the phenomenon could permit a greater understanding of the events necessary for the initiation of DNA synthesis.

Another feature of the cell cycle of this organism, which is useful in some respects, is the shortness of the S phase. It has the disadvantage that it makes the analysis of the synthetic process difficult, but in relation to the other events it provides a distinct marker to which they can be related. Because the initiation and completion of DNA synthesis are so close in time the S phase can virtually be considered as a single event, which shows very little overlap in the whole cycle with the other synthetic processes. This feature accentuates the difference between the chemical replication of genes (DNA) and the doubling of the gene products, and clearly shows that there is a delay between them.

The measurement of various gene products in S. pombe has

been discussed in the introduction in chapter 1, but mention of some of them could be made again to illustrate this intriguing phenomenon. Two biosynthetic enzymes, aspartate transcarbamylase and ornithine transcarbamylase, are synthesised in short bursts at 0.4 of the way through the cell cycle, whereas tryptophan synthetase is synthesised in a burst at 0.9 of the way through the cycle. The enzymes, sucrase, alkaline phosphatase and acid phosphatase, are synthesised continuously throughout the cycle though they show a discrete doubling in the rate of synthesis at 0.4 of the way through the cycle. The rate of synthesis of induced sucrase also doubles at 0.4 of the way through the cell cycle (Mitchison, 1967). With the exception of tryptophan synthetase all the enzymes studied so far double in quantity or in the rate of synthesis at a time which is intermediate between DNA synthesis and nuclear division. There are basically two processes which take place before the information contained in the DNA code is expressed in the form of a protein. These are transcription and translation, and there must be a delay in one or both of these before information is expressed in S. pombe. The separation of the chemical replication of a gene and the doubling in the quantity of its product has been shown before (e.g. Tauro et al., 1968; Masters and Donachie, 1966), but S. pombe also shows the separation of the chemical replication of a gene and the doubling in the rate of synthesis of its product, which, in the bacteria, for example, are very closely related in time

(reviewed in Donachie and Masters, 1968).

The other aspect of this phenomenon is the relationship between the timing of DNA synthesis and the behaviour of cells following ultraviolet irradiation, and their resistance to the damage to DNA due to 32P decays. Cells are extremely resistant to killing by ultraviolet light and 32P decays between 0.4 and 0.6 of the way through the cell cycle, whereas they are highly sensitive to these treatments between 0.9 of one cycle and 0.2 in the next (Swann, 1962). The change from extreme sensitivity to resistance coincides with the point in the cell cycle at which there is a change in the distribution of deaths of daughter cells following irradiation of the parent cell. Irradiation of cells from 0.0 to 0.15 in the cycle yielded a 1:1 ratio of similar and differential deaths in the progeny of the irradiated cells, whereas irradiation after 0.45 of the way through the cell cycle yielded almost completely differential deaths in the daughter cells (Swann, 1962). The simplest interpretation of these results, and the one that is put forward by Swann (1962), is that the rise in resistance to killing and the changeover from similar to differential daughter deaths represent the time at which 'gene' (DNA) replication takes place. It clearly is not the chemical replication of the gene in terms of its DNA content, but what precisely the ultraviolet irradiation experiments are measuring is not possible to say at the present time. The effects of ultraviolet light are complex

and numerous. It can cause modifications in the bases within DNA (reviewed by Shooter, 1967), produce cross-links between different strands of the DNA (Marmur and Grossman, 1961), and form cross-links between DNA and protein (Smith *et al.*, 1966). Added to these, damage due to ultraviolet light can be repaired by enzyme systems which have been shown to be present in *S. pombe* (Clarke, 1968). More information is needed about the processes involved in ultraviolet light and ^{32}P-decay damage before it would be possible to identify the component of a 'gene' that these treatments are measuring.

The results presented in this thesis are essentially a description of the DNA synthesis cycle and give little information on the mechanisms that control the initiation and maintenance of DNA synthesis. They do show that the initiation of the synthesis phase can be displaced in the cell cycle and an analysis of this phenomenon could yield information about the processes necessary for the start of DNA synthesis. These results also suggest that, in this organism, once the S phase has started it is always carried to completion in approximately the same length of time that it is when the cells are grown under optimal conditions. This raises the question of whether it is possible for initiation to occur without the completion of the S phase, and whether the conditions necessary for the initiation of S are sufficient to ensure the completion of the S phase. Within the broader context of the control of other

macromolecular syntheses these results raise the question of what is causing the delay between the replication of the DNA within the genome and the doubling in the gene product. The knowledge of the pattern of DNA synthesis in S. pombe has added to our knowledge of this organism's cell cycle, but in doing so it has raised several further questions related to the control of macromolecular synthetic pathways.

Summary

Estimations of the mean DNA content of cells of Schizosaccharomyces pombe at various stages of the growth cycle were made. From these it was found that lag phase cells contain 0.0292 µµg. DNA per cell, log. phase cells contain 0.0376 µµg. DNA per cell, stationary phase cells, in Edinburgh Minimal Medium 1, contain 0.0199 µµg. DNA per cell and spores contain 0.0146 µµg. DNA per spore.

From these results it was suggested that the pattern of DNA synthesis in the cell cycle of S. pombe is as follows. The synthesis of DNA is fairly rapid, of the order of 15 minutes, and occurs immediately after nuclear fission and migration. The cell cycle is therefore predominantly composed of G2, there being very little G1.

This pattern of DNA synthesis was subsequently confirmed by an analysis of synchronously dividing cell cultures.

A study of the amount of mitochondrial DNA present in cells during log. and stationary growth phases showed that the proportion of mitochondrial DNA increased as the cells passed into stationary phase. During logarithmic growth about 1% of the total DNA was mitochondrial DNA whereas in stationary phase this increased to 8%.

The mean DNA content of stationary phase cells suggested

that, under the limiting conditions present at that stage in the growth cycle, a significant G1 phase could be inserted into the cell cycle.

Experiments involving variations in the level of phosphate in the medium showed that it was possible to determine whether cells would enter stationary phase in G1 or G2. By growing the cells in a chemostat under conditions of limiting phosphate the cell cycle could be altered so that the majority of it was the G1 phase.

Variations in the growth rate brought about by alteration of the growth temperature, or substitution of glycerol for glucose in the medium, lengthened each DNA synthesis phase in proportion to the increase in the total generation time.

The effect of the DNA synthesis inhibitor, 2-phenyl ethanol, was investigated and it was shown that it had an unique effect on S. pombe. At a concentration of 0.2% of 2-phenyl ethanol the cell cycle was changed so that a G1 phase occupied approximately half of the total cycle. Cells could be cultured indefinitely in medium containing this concentration of the inhibitor.

These results were discussed in relation to other work on the fission yeast and the DNA synthetic patterns in other organisms.

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References

Abbo, F.E. and Pardee, A.B., 1960. Synthesis of macromolecules in synchronously dividing bacteria. Biochim. biophys. Acta 39 478.

Arber, W., 1965. Host specificity of DNA produced by E. coli. V. The role of methionine in the production of host specificity. J. molec. Biol. 11 247.

Barner, H.D. and Cohen, S.S., 1956. Synchronisation of division of a thymineless mutant of E. coli. J. Bact. 72 115.

Basserga, R., Estensen, R.D. and Peterson, R.O., 1965. Inhibition of DNA synthesis in Ehrlich Ascites cells by Actinomycin D. II. The presynthetic block in the cell cycle. Proc. natn. Acad. Sci. U.S.A. 54 1141.

Berrah, G. and Konetzka, W.A., 1962. Selective and reversible inhibition of the synthesis of bacterial DNA by phenethyl alcohol. J. Bact. 83 738.

Billen, D., 1968. Methylation of bacterial chromosomes: an event at the 'replication point'? J. molec. Biol. 31 477.

Bitten, D. and Hnilica, L.S., 1964. Inhibition of DNA synthesis by histones. In: The Nucleohistones, ed. J. Bonner and P. Ts'o. Holden-Day Inc. San Fran., Lon. and Amster.

Bostock, C.J., Donachie, W.D., Masters, M. and Mitchison, J.M., 1966. Synthesis of enzymes and DNA in synchronous cultures of Schizosaccharomyces pombe. Nature 210 808.

Bruchovsky, N. and Till, J.E., 1967. Effects of β -phenethyl alcohol on mouse L cells in suspension culture. 1. Reversible inhibition of cell proliferation and effects on the uptake of labelled precursors of nucleic acids and protein. Mol. pharmacol. 3 124.

Burton, K., 1956. A study of the conditions and mechanisms of the diphenylamine reaction for the colourimetric estimation of deoxyribonucleic acid. Biochem. J. 62 315.

Cameron, I.L., 1964. Is the duration of DNA synthesis in somatic cells of mammals and birds constant? J. Cell Biol. 20 185.

Cameron, I.L. and Nachtwey, D.S., 1967. DNA synthesis in relation to cell division in Tetrahymena pyriformis. Expl. Cell Res. 46 385.

Cameron, I.L. and Stone, G.E., 1964. Relation between the amount of DNA per cell and the duration of DNA synthesis in three strains of Tetrahymena pyriformis. Expl. Cell Res. 36 510.

Clarke, C.H., 1968. Differential effects of caffeine in mutagen treated Schizosaccharomyces pombe. Mut. Res. 5 33.

Clarke, D.J. and Maaløe, O., 1967. DNA replication and the division cycle in E. coli. J. molec. Biol. 23 99.

Cleaver, J.E., 1967. Thymidine Metabolism and Cell Kinetics. North Holland Monographs, Frontiers of Biology 6, ed. A. Neuberger and E.L. Tatum. Amster.

Cleffman, G., 1967. Temperaturabhangigkeit der Phasen des Teilungszyklus von Tetrahymena pyriformis H.S.M. Z. Zellforsch. 79 599.

Clowes, F.A.L., 1965. The duration of the G1 phase of the mitotic cycle and its relation to radiosensitivity. New Phytol. 64 355.

Cohen, S.S., Flaks, J.G., Barner, H.D., Lock, M.R. and Lichenstein, J., 1958. The mode of action of 5-fluorouracil and its derivatives. Proc. nat. Acad. Sci. U.S.A. 44 1004.

Cooper, S. and Helmstetter, C.E., 1968. Chromosome replication and the division cycle of Escherichia coli B/r. J. molec. Biol. 31 519.

Corneo, G., Moore, C., Sanadi, R., Grossman, L.I. and Marmur, J., 1966. Mitochondrial DNA in yeast and some mammalian species. Science 151 687.

Croes, A.F., 1966. Duplication of DNA during meiosis in Baker's yeast. Exp. Cell Res. 41 452.

Cummins, J.E. and Rusch, H.P., 1966. Limited DNA synthesis in the absence of protein synthesis in Physarum polycephalum. J. Cell Biol. 31 577.

Cummins, J.E. and Mitchison, J.M., 1964. A method for making autoradiographs of yeast cells which retain pool components. Exp. Cell Res. 34 406.

Cutler, R.G. and Evans, J.E., 1966. Synchronisation of bacteria by a stationary phase method. J. Bact. 91 469.

Dalcq, A. and Pasteels, J., 1955. Determination photometrique de la teneur relative en DNA des noyaux dans les oeufs en segmentation du rat et de la souris. Exp. Cell Res. Suppl. 3 72.

Defendi, V. and Manson, L.A., 1963. Analysis of the life cycle in mammalian cells. Nature 198 359.

Donachie, W.D., 1965. Control of enzyme steps during the bacterial cell cycle. Nature 205 1084.

Donachie, W.D. and Masters, M., 1968. Temporal control of gene expression in bacteria. In: 'Cell cycle: genetic and developmental aspects,' ed. I.L. Cameron, G.D. Padilla and G.L. Whitson. Acad. Press, New York and London.

Doskocil, J. and Sorm, F., 1962. Distribution of 5-methyl-cytosine in pyrimidine sequences of deoxyribonucleic acid. Biochim. biophys. Acta 55 953.

Eaton, N.R., 1962. New press for disruption of micro-organisms. J. Bact. 83 1359.

Eburle, H. and Lark, K.G., 1967. Chromosome replication in Bacillus subtilis cultures growing at different rates. Proc. nat. Acad. Sci. U.S.A. 57 95.

Eddy, A.A. and Williamson, D.H., 1957. A method of isolating protoplasts from yeast. Nature 179 1252.

Evans, H.J., 1964. Uptake of 3H-thymidine and patterns of DNA replication in nuclei and chromosomes of Vicia faba. Exp. Cell Res. 35 381.

Faed, M.J.W., 1959. Ph.D. Thesis, University of Edinburgh.

Ferretti, J. and Gfay, G.D., 1967. Control of enzyme synthesis during adaptation in synchronously dividing populations of Rhodopseudomonas spheroides. Biochem. biophys. Res. commun. 29 501.

Flamm, W.G., Birnstiel, M.L. and Walker, P.M.B., 1968. In: 'Subcellular components: preparation and fractionation,' ed. G.D. Birnie and S.M. Fox. Butterworths, London.

Flickinger, C.J., 1967. The fine structure of the nuclei of Tetrahymena pyriformis throughout the cell cycle. J. Cell Biol. 27 519.

Freese, E.B. and Freese, E., 1963. The rate of DNA strand separation. Biochemistry 2 707.

Fujimoto, D., Srinivasan, P.R. and Borek, E., 1965. On the nature of deoxyribose nucleic acid methylases. Biological evidence for the multiple nature of the enzymes. Biochemistry 4 2849.

Gabriel, L., 1965. Inhibition of growth, synthesis and permeability in Neurospora crassa by phenethyl alcohol. J. Bact. 90 29.

Ganesan, A.T. and Swaminathan, M.S., 1958. Staining the nucleus in yeasts. Stain Technol. 33 115.

Gaulden, M.E., 1956. DNA synthesis and X-ray effects at different mitotic stages in grasshopper neuroblasts. Genetics 41 645.

Gelfant, S., 1963. In: Cell Growth and Cell Division, vol. 2, ed. R.J.C. Harris. Acad. Press, New York and London.

Giles, K.W. and Myers, A., 1965. An improved diphenylamine method for the estimation of deoxyribonucleic acid. Nature 206 93.

Gill, B.F., 1965. Ph.D. Thesis, University of Edinburgh.

Gorman, J., Tauro, P., LaBerge, M. and Halvorson, H., 1964. Timing of enzyme steps during synchronous division in yeast. Biochem. biophys. Res. commun. 15 43.

Graham, C.F. and Morgan, R.W., 1966. Changes in the cell cycle during early amphibian development. Devel. Biol. 14 439.

Gross, W.A., Deitz, W.H. and Cook, T.M., 1964. Mechanism of action of Nalidixic acid on Escherichia coli. J. Bact. 88 1112.

Grunberg-Manago, M., 1963. In: Progress in Nucleic Acid Research, vol. 1, ed. J.N. Davidson and W.E. Cohn. Acad. Press, New York and London.

Harris, H., 1968. Nucleus and Cytoplasm. Clarendon Press, Oxford.

Helmstetter, C.E., 1967. Rate of DNA synthesis during the division cycle of Escherichia coli B/r. J. molec. Biol. 24 417.

Helmstetter, C.E. and Cooper, S., 1968. DNA synthesis during the division cycle of rapidly growing Escherichia coli B/r. J. molec. Biol. 31 507.

Herring, A.J. Personal communication.

Hindegardner, R.T., Rao, B. and Feldman, D.C., 1964. The DNA synthetic period during early development of the sea urchin egg. Exp. Cell Res. 36 53.

Howard, A. and Pelc, S.R., 1953. Synthesis of deoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. Heredity (suppl.) 6 261.

Hughes, A., 1952. The mitotic cycle; the cytoplasm and nucleus during interphase and mitosis. Butterworths, London.

Hutchinson, W.C. and Munro, H.N., 1961. Determination of nucleic acids in biological materials. A review. The Analyst 86 768.

Ingram, M., 1955. An introduction to the biology of yeasts. Pitman, London.

Iyer, V.N. and Szybalski, W., 1963. A molecular mechanism of mitomycin action: linking of complementary DNA strands. Proc. nat. Acad. Sci. U.S.A. 50 355.

Jacob, F., Brenner, S. and Curzin, F., 1963. On the regulation of deoxyribonucleic acid replication in bacteria. Cold Spring Harb. Symp. Quant. Biol. 28 329.

Jacob, F. and Monod, J., 1963. In: "Cytodifferentiation and macromolecular synthesis," ed. M. Locke, 21st Symp. of the Society for the Study of Development and Growth. Acad. Press, New York and London.

Johnson, B.F., 1968a. Morphometric analysis of yeast cells. II. Cell size of Schizosaccharomyces pombe during the growth cycle. Exp. Cell Res. 49 59.

Johnson, B.F., 1968b. Lysis of yeast cell walls induced by 2-deoxyglucose at their sites of glucan synthesis. J. Bact. 95 1169.

Kantor, G.H. and Deering, R.A., 1968. Effect of nalidixic acid and hydroxyurea on division ability of Escherichia coli fil$^+$ and lon$^-$ strains. J. Bact. 95 1968.

Killander, D. and Zetterberg, A., 1965. Quantitative cytochemical studies on interphase growth. 1. Determination of DNA, RNA and mass content of age determined mouse fibroblasts in vitro and of intercellular variation in generation time. Exp. Cell Res. 38 272.

Kimball, R.F. and Barka, T., 1959. Quantitative cytochemical studies on Paramecium aurelia. II. Feulgen microspectrophotometry of the macronucleus during exponential growth. Exp. Cell Res. 17 173.

Kimball, R.F. and Purdue, S.W., 1962. Quantitative cytochemical studies of nucleic acid synthesis. Exp. Cell Res. 17 405.

Kimball, R.F. and Vogt-Kohne, L., 1961. Quantitative cytochemical studies on Paramecium aurelia. IV. The effect of limited food and starvation on the macronucleus. Exp. Cell Res. 23 479.

Kirby, K.S., 1964. In: 'Progress in nucleic acid research and molecular biology,' ed. J.N. Davidson and W.E. Cohn. Acad. Press, New York and London.

Kissane, J.M. and Robbins, E., 1958. The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. J. Biol. Chem. 233 184.

Kjeldgaard, N.O., Maaløe, O. and Schaechter, M., 1958. The transition between different physiological states during balanced growth in Salmonella typhimurium. J. Gen. Microbiol. 19 607.

Lark, C., 1966. Regulation of deoxyribonucleic acid synthesis in Escherichia coli: dependence of growth rates. Biochim. biophys. Acta 119 517.

Lark, C., 1968a. Studies on the in vivo methylation of DNA in Escherichia coli 15T$^-$. J. molec. Biol. 31 389.

Lark, C., 1968b. Effect of the methionine analogs, ethionine or norleucine, on DNA synthesis in Escherichia coli 15T$^-$. J. molec. Biol. 31 389.

Lark, K.G., 1966. Regulation of chromosome replication and segregation in bacteria. Bact. Rev. 30 3.

Lark, K.G. and Lark, C., 1966. Regulation of chromosome replication in Escherichia coli: a comparison of the effects of phenethyl alcohol treatment with those of amino acid starvation. J. molec. Biol. 20 9.

Lawley, P.D., 1966. In: 'Progress in Nucleic Acid Research and Molecular Biology,' vol. 5, ed. J.N. Davidson and W.E. Cohn. Acad. Press, New York and London.

Leach, F.R., Best, N.H., Davis, E.M., Saunders, D.C. and Gimlin, D.M., 1964. Effect of phenethyl alcohol on cell growth. Exp. Cell Res. 36 527.

Lee, J.B., 1963. Compounds interfering with the diphenylamine test for deoxyribonucleic acid. Nature 200 264.

Leupold, U., 1956. Some data on polyploid inheritance in Schizosaccharomyces pombe. C-R. Lab. Carles. Ser. physiol. 26 221.

Littlefield, J.W. and Jacob, P.S., 1965. The relation between deoxyribonucleic acid and protein synthesis in mouse fibroblasts. Biochim. biophys. Acta 108 652.

Loveless, A., 1966. Genetic and allied effects of alkylating agents. Butterworth, London.

MacDonald, B.B., 1958. Quantitative aspects of deoxyribonucleic acid metabolism in an amicronucleate strain of Tetrahymena. Biol. Bull. 114 71.

MacDonald, B.B., 1960. Time of deoxyribonucleic acid synthesis in micro- and macronuclei of Tetrahymena pyriformis. J. Protozool. 7 (suppl.) 10.

MacKenzie, T.B., Stone, G.E. and Prescott, D.M., 1966. The durations of G1, S, and G2 at different temperatures in Tetrahymena pyriformis, H.S.M. J. Cell Biol. 31 633.

Malek, I. and Sencl, Z., 1966. Theoretical and methodological basis of continuous culture of micro-organisms. Acad. Press, New York and London.

Marchok, A., 1965. Cell division and nucleic acid synthesis in cells of developing chick muscle. J. Cell Biol. 27 63A.

Marmur, J. and Grossman, L., 1961. Ultraviolet light induced linking of deoxyribonucleic acid and its reversal by photoreactivating enzymes. Proc. nat. Acad. Sci. U.S.A. 47 778.

Maruyama, Y., 1956. Biochemical aspects of the cell growth of Escherichia coli as studied by the method of synchronous culture. J. Bact. 72 821.

Maruyama, Y. and Lark, K.G., 1962. Periodic nucleotide synthesis in synchronous cultures of bacteria. Exp. Cell Res. 26 382.

Masters, M. and Donachie, W.D., 1966. Repression and the control of cyclic enzyme synthesis in Bacillus subtilis. Nature 209 476.

McConkey, E.H. and Hopkins, J.W., 1964. The relationship of the nucleolus to the synthesis of ribosomal RNA in HeLa cells. Proc. nat. Acad. Sci. U.S.A. 51 1197.

Mendelson, N.H. and Frazer, D., 1965. Physical effects of the inhibitor β -phenethyl alcohol. Biochim. biophys. Acta 102 559.

Meselson, M. and Yuan, R., 1968. DNA restriction enzyme from E. coli. Nature 217 1110.

Mitchison, J.M., 1957. The growth of single cells. 1. Schizosaccharomyces pombe. Exp. Cell Res. 13 244.

Mitchison, J.M., 1963a. The cell cycle of a fission yeast. Symp. Soc. Cell Biol. 2 151.

Mitchison, J.M., 1963b. Patterns of synthesis of RNA and other cell components during the cell cycle of Schizosaccharomyces pombe. J. Cell Comp. Physiol. 62 (suppl. 1) 1.

Mitchison, J.M., 1967. In: Abstracts of VIIth International Congress of Biochemistry, p. 363.

Mitchison, J.M. and Cummins, J.E., 1964. Changes in the acid-soluble pool during the cell cycle of Schizosaccharomyces pombe. Exp. Cell Res. 35 394.

Mitchison, J.M. and Gross, P.R., 1965. Selective synthesis of messenger RNA in a fission yeast during a stepdown and its relation to the cell cycle. Exp. Cell Res. 37 259.

Mitchison, J.M., Kinghorn, M.L. and Hawkins, C., 1963. The growth of single cells. IV. Schizosaccharomyces pombe at different temperatures. Exp. Cell Res. 30 521.

Mitchison, J.M. and Lark, K.G., 1962. Incorporation of 3H-adenine into RNA during the cell cycle of Schizosaccharomyces pombe. Exp. Cell Res. 28 452.

Mitchison, J.M. and Vincent, W.S., 1965. Preparation of synchronous cell cultures by sedimentation. Nature 205 987.

Mitchison, J.M. and Wilbur, K.M., 1962. The incorporation of protein and carbohydrate precursors during the cell cycle of a fission yeast. Exp. Cell Res. 26 144.

Mittermayer, C., Braun, R. and Rusch, H.P., 1964. RNA synthesis in the mitotic cycle of Physarum polycephalum. Biochim. biophys. Acta 91 399.

Moses, M.J. and Taylor, J.H., 1955. Desoxyribose nucleic acid synthesis during microsporogenesis in Tradescantia. Exp. Cell Res. 9 474.

Moustacchi, E. and Williamson, D.H., 1966. Physiological variations in satellite components of yeast DNA detected by density gradient centrifugation. Biochem. biophys. Res. commun. 23 56.

Nass, M.M.K., Nass, S. and Afzelius, A., 1965. The general occurrence of mitochondrial DNA. Exp. Cell Res. 37 516.

Nygaard, O.F., Guttes, S. and Rusch, H.P., 1960. Nucleic acid metabolism in a slime mould with synchronous mitosis. Biochim. biophys. Acta 38 298.

Prescott, D.M., 1960. Relation between cell growth and cell division. IV. The synthesis of DNA, RNA and protein from division to division in Tetrahymena. Exp. Cell Res. 19 228.

Prescott, D.M., 1964. In: 'The Nucleohistones,' ed. J. Bonner and P. Ts'o. Holden-Day Inc., San Fran., London and Amster.

Prescott, D.M., 1966. The synthesis of total macromolecular protein, histone and DNA during the cell cycle in Euplotes eurystomus. J. Cell Biol. 31 1.

Prescott, D.M. and Carrier, R.F., 1964. In: 'Methods in Cell Physiology,' vol. 1, ed. D.M. Prescott. Acad. Press, New York and London.

Prescott, D.M. and Goldstein, L., 1967. Nuclear-cytoplasmic interaction in DNA synthesis. Science 155 469.

Prescott, D.M. and Kimball, R.F., 1961. Relation between RNA, DNA and syntheses in the replicating nucleus of Euplotes. Proc. nat. Acad. Sci. U.S.A. 47 686.

Prescott, D.M. and Stone, G.E., 1967. In: 'Research in Protozoology,' vol. 2, ed. Tze-Tuan Chen. Pergammon.

Provost, C. and Moses, V., 1966. Action of phenethyl alcohol on the synthesis of macromolecules in Escherichia coli. J. Bact. 91 1446.

Rao, P.N. and Engelberg, J., 1966. Effects of temperature on the mitotic cycle of normal and synchronised mammalian cells. In: 'Cell Synchrony,' ed. I.L. Cameron and G.M. Padilla. Acad. Press, New York and London.

Reddan, J.R. and Rothstein, H., 1967. Growth dynamics of an amphibian tissue. J. Cell Comp. Physiol. 67 307.

Richards, B.M., Walker, P.M.B. and Deely, E.M., 1956. Changes in nuclear DNA in normal and Ascites tumour cells. Ann. N.Y. Acad. Sci. 63 831.

Robbins, E. and Scharff, M.D., 1967. The absence of a detectable G1 in a cultured strain of Chinese hamster lung cells. J. Cell Biol. 34 684.

Robinow, C.F. and Bakerspiegel, A., 1965. In: 'The Fungi,' ed. G.C. Ainsworth and S. Sussman, p. 119. Acad. Press, New York and London.

Rosencranz, H.S., Carr, H.S. and Rose, H.M., 1965. Phenethyl alcohol. II. Effect on thymine requiring Escherichia coli. J. Bact. 89 1370.

Rupp, W.D. and Howard-Flanders, P., 1968. Discontinuities in the DNA synthesised in an excision defective strain of Escherichia coli following ultraviolet irradiation. J. Molec. Biol. 31 291.

Sachsenmaier, W. and Rusch, H.P., 1964. The effect of 5-fluoro-2-deoxyuridine on synchronous mitosis in Physarum polycephalum. Exp. Cell Res. 36 124.

Robinson, A.R., 1966. B.Sc. Honours Thesis, Department of Zoology, University of Edinburgh.

Sando, N., 1963. Biochemical studies on the synchronised culture of Schizosaccharomyces pombe. J. Gen. Appl. Microbiol. 9 233.

Schaechter, M., Maaløe, O. and Kjeldgaard, N.O., 1958. Dependency on medium and temperature of cell size and chemical composition during balanced growth of Salmonella typhimurium. J. Gen. Microbiol. 19 592.

Schaechter, M., Bentzon, M.W. and Maaløe, O., 1959. The synthesis of DNA during the division cycle of bacteria. Nature 183 1207.

Scharff, M.D. and Robbins, E., 1965. Synthesis of ribosomal RNA in synchronised HeLa cells. Nature 208 464.

Schmidt, R.E., 1966. In: 'Cell Synchrony,' ed. I.L. Cameron and G.M. Padilla. Acad. Press, New York and London.

Schmitter, R. 1966. Ph.D. Thesis, University of Edinburgh.

Sherman, F.G., Quastler, H. and Wimber, D.R., 1961. Cell population kinetics in the ear epidermis of mice. Exp. Cell Res. 25 114.

Shooter, K.V., 1967. 'The effects of radiation on DNA biosynthesis and related processes,' in Prog. Biophys. Mol. Biol., vol. 17 289.

Sinclair, W.K., 1965. Hydroxyurea: differential lethal effects on cultured mammalian cells during the cell cycle. Science 150 1729.

Sisken, J.E., 1965. Exposure time as a parameter in the effects of temperature on the duration of metaphase. Exp. Cell Res. 40 436.

Sisken, J.E. and Kinosita, R., 1961. The timing of DNA synthesis in the mitotic cycle in vitro. J. Biophys. Biochem. Cytol. 9 509.

Sisken, J.E. and Morasca, L., 1965. Intrapopulation kinetics of the mitotic cycle. J. Cell Biol. 25 (suppl.) 179.

Sisken, J.E., Morasca, L. and Kibby, S., 1965. Effects of temperature on the kinetics of the mitotic cycle of mammalian cells in culture. Exp. Cell Res. 39 103.

Slepecky, R.A. and Celkis, Z., 1964. Inhibition of bacterial sporulation and germination by phenethyl alcohol and related compounds. Bact. Proc. 64 14.

* Schneider (1945), see next page.

Smith, G.M., 1955. 'Cryptogamic Botany,' vol. 1. McGraw-Hill, New York, Toronto and London.

Smith, K.C., Hodgkins, B. and O'Leary, M.E., 1966. The biological importance of ultraviolet light induced DNA-protein crosslinks in Escherichia coli 15 TAU. Biochim. biophys. Acta 114 1.

Schneider, W.C., 1945. Phosphorus compounds in animal tissues. 1. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem. 161 293

Southern, E. Personal communication.

Srinivasan, P.R. and Borek, E., 1964. Enzymic alteration of nucleic acid structure. Science 145 548.

Stebbing, N. Personal communication.

Steinberg, W. and Halvorson, H.O., 1968. Timing of enzyme synthesis during outgrowth of spores of Bacillus cereus. II. Relationship between ordered enzyme synthesis and DNA replication. J. Bact. 95 479.

Steinberg, W., Halvorson, H.O., Keynan, A. and Weinberg, E., 1965. Timing of protein synthesis during germination and outgrowth of spores of Bacillus cereus strain T. Nature 208 710.

Stone, G.E. and Cameron, I.L., 1964. In: 'Methods in Cell Physiology,' ed. D.M. Prescott. Acad. Press, New York and London.

Stone, G.E. and Prescott, D.M., 1964. Cell division and DNA synthesis in Tetrahymena pyriformis deprived of essential amino acids. J. Cell Biol. 21 275.

Stonehill, E.H. and Hutchinson, D.J., 1966. Chromosomal mapping by means of mutational induction in synchronous populations of Streptococcus faecalis. J. Bact. 92 136.

Swann, M.M., 1957. The control of cell division. 1. General mechanisms. Cancer Research 17 727.

Swann, M.M., 1962. Gene replication, ultraviolet sensitivity and the cell cycle. Nature 193 1222.

Swann, M.M. Personal communication.

Tauro, P., Halvorson, H.O. and Epstein, R.L., 1968. Time of gene expression in relation to centromere distance during the cell cycle of Saccharomyces cerevisiae. Proc. nat. Acad. Sci. U.S.A. 59 271.

Taylor, J.H., 1960. Nucleic acid synthesis in relation to the cell division cycle. Ann. N.Y. Acad. Sci. 90 409.

Taylor, J.H., 1963. Chapter II in 'Molecular Genetics,' ed. J.H. Taylor. Acad. Press, New York and London.

Terasima, T. and Tolmach, L.J., 1963. Growth and nucleic acid synthesis in synchronously dividing populations of HeLa cells. Exp. Cell Res. 30 344.

Tewari, K.K., Votsch, W., Mahler, H.R. and Mackler, B., 1966. Biochemical correlates of respiratory deficiency. VI. Mitochondrial DNA. J. molec. Biol. 20 453.

Till, J.E., Whitmore, G.F. and Gulyas, S., 1963. Deoxyribonucleic acid synthesis in individual L strain mouse cells. II. Effects of thymidine starvation. Biochim. biophys. Acta 72 277.

Trieck, R.W., 1966. Control of DNA synthesis in Escherichia coli by phenethyl alcohol. Diss. Abstr. 26 6313.

V'ant Hof, J., 1966. Comparative cell population kinetics of tritiated thymidine labelled diploid and colchicine induced tetraploid cells in the same tissue of Pisium. Exp. Cell Res. 41 274.

Vincent, W.S., 1965. Cyclical synthesis of ribosomes in synchronous cell cultures. J. Cell Biol. 27 109A.

Von Borstal, R.C., 1966. Microbial Genetics Bull. 25 (suppl.).

Waring, M.J., 1966. In: 'Biochemical Studies of Antimicrobial Drugs,' 16th Symp. Soc. Gen. Microbiol., ed. B.A. Newton and P.E. Reynolds.

Watambe, I. and Okada, S., 1967. Effects of temperature on growth rateof cultured mammalian cells (L. 51784). J. Cell Biol. 32 309.

Williamson, D.H., 1965. The timing of deoxyribonucleic acid synthesis in the cell cycle of Saccharomyces cerevisiae. J. Cell Biol. 25 517.

Williamson, D.H., 1966. In: 'Cell Synchrony,' ed. I.L. Cameron and G.M. Padilla. Acad. Press, New York and London.

Williamson, D.H. and Scopes, A.W., 1960. The behaviour of nucleic acids in synchronously dividing cultures of Saccharomyces cerevisiae. Exp. Cell Res. 20 339.

Williamson, D.H. and Scopes, A.W., 1961. The distribution of nucleic acids and protein between different sized yeast cells. Exp. Cell Res. 24 151.

Wilson, B.W. and Levedahl, B.H., 1964. Synthetic and division rates of Euglena gracilis grown in batch cultures. Exp. Cell Res. 35 69.

Wimber, D.E., 1966. Duration of the nuclear cycle in Tradescantia root tips at three temperatures as measured with 3H-thymidine. Amer. J. Bot. 53 21.

Wimber, D.E. and Quastler, H., 1963. A 14C and 3H-thymidine double labelling technique in the study of cell proliferation in Tradescantia root tips. Exp. Cell Res. 30 8.

Yoshikawa, H. and Sueoka, N., 1963. Sequential replication of Bacillus subtilis chromosome. I. Comparison of marker frequencies in exponential and stationary growth phases. Proc. nat. Acad. Sci. U.S.A. 49 559.

Yotsuyanagi, Y., 1962. Etudes sur le chondriome de levure. (I). Variation de l'ultrastructure du chondriome au cours du cycle de la croissance aerobie. (II). Chondriomes de mutants a deficience respiratoire. J. Ultrastruct. Res. 7 121 and 141 respectively.

Young, R.W., 1962. Regional differences in cell generation time in growing rat tibiae. Exp. Cell Res. 26 562.

Young, I.E. and Fitzjames, P.C., 1959. Patterns of synthesis of deoxyribonucleic acid in Bacillus cereus growing synchronously out of spores. Nature 183 372.

Zeuthen, E., 1963. In: 'Cell Growth and Cell Division,' ed. R.J.C. Harris. Acad. Press, New York and London.

Appendix 1

Culture Media

The following were the composition of the media:

Edinburgh Minimal Medium 1 - EMM 1 - for Schizosaccharomyces pombe. (Modified from minimal medium in Leupold, U., Arch. Jul. Klaus-Stiftung, Zurich 30 506 (1955).)

In 1000 ml. water:

(A)	C - source	Glucose	10 g.
(B)	N - source	NH_4Cl	5 g.
(C)	Salts	Sodium acetate (buffer)	1 g.
		KCl	1 g.
		$MgCl_2$	0.5 g.
		NaH_2PO_4	10 mg.
		Na_2SO_4	10 mg.
		$CaCl_2$	10 mg.
(D)	Vitamins	Inositol	10 mg.
		Nicotinic acid	10 mg.
		Calcium pantothenate	1 mg.
		Biotin	10 µg.
(E)	Trace elements	H_3BO_3	500 µg.
		$MnSO_4.H_2O$	400 µg.
		$ZnSO_4.7H_2O$	400 µg.
		$FeCl_3.6H_2O$	200 µg.
		$H_2MoO_4.H_2O$	160 µg.
		KI	100 µg.
		$CuSO_4.5H_2O$	40 µg.
		Citric acid	1000 µg.

The pH of this medium is about 5.3 before growth of cells, but is reduced to about 4.6 in stationary phase cultures.

EMM 2 is the same except that the phosphate concentration is 30X higher - i.e. 300 mg./L. This should be added separately to the final solution. EMM 2 gives a higher final yield of cells and prevents phosphate depletion of the medium.

Malt Extract Agar

In 1000 ml. distilled:

30 gms. of 'Oxoid' Malt Extract

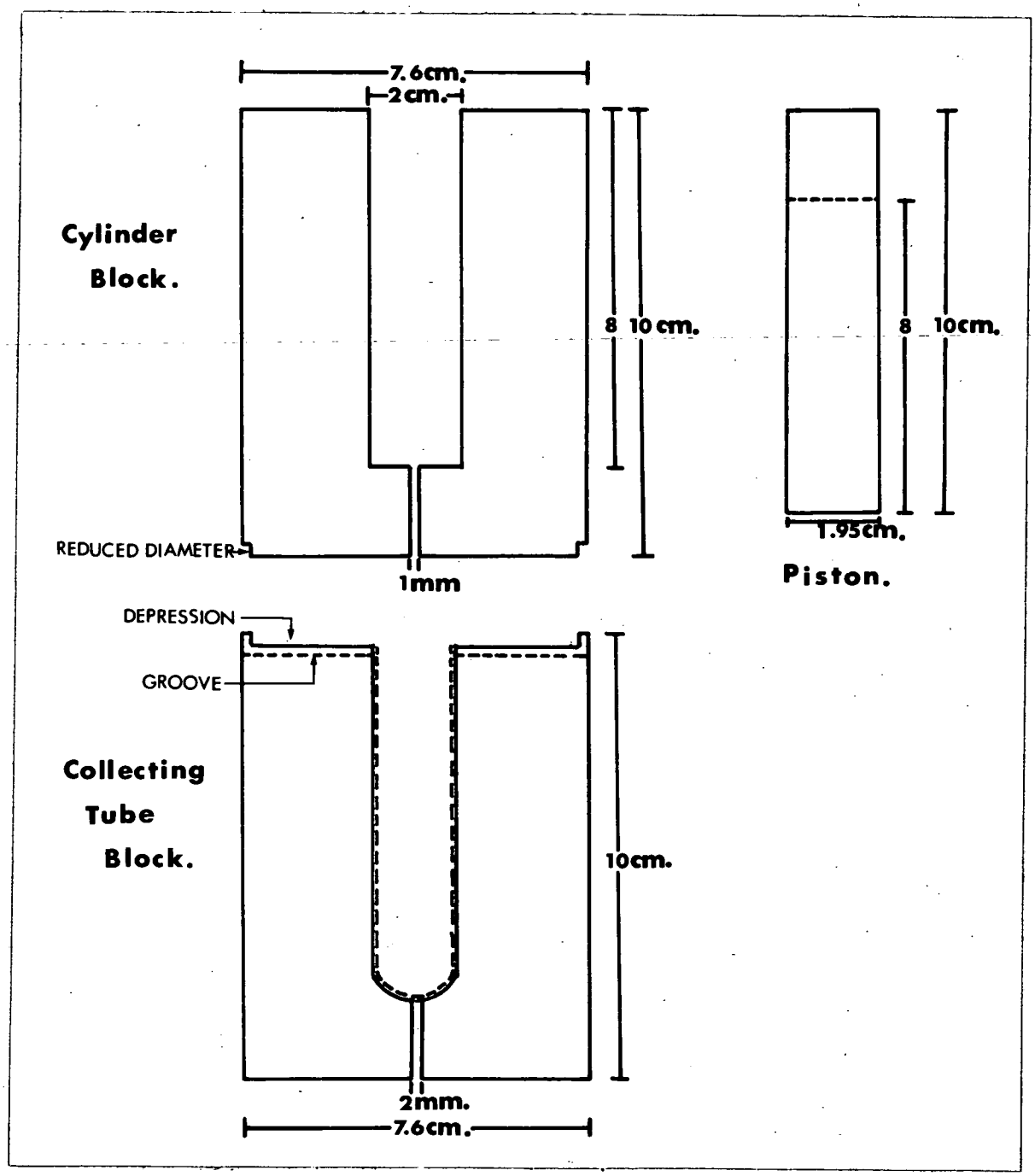
15 gms. powdered bacteriological agar

Appendix 2

The Eaton Press

The press used was a modification of that described originally by Eaton (1962). The cylinder block and the receiving tube block were machined from a cylindrical bar of stainless steel as shown in figure 39. The cylinder block had a central hole, 2 cm. in diameter, into which fitted the piston, except for the bottom 2 cm. In this region there was a small hole, 1 mm. in diameter, through which passed the cell suspension during operation of the press. The piston was machined from stainless steel, hardened and ground to a diameter 0.050 cm. less than the diameter of the cylinder. The bottom of the cylinder block had a reduced diameter which fitted into a recess in the top of the receiving tube block. The latter had a central hole which would just take a 10 ml. stainless steel MSE centrifuge tube. There were grooves cut radially in the top of the receiving tube block, and a 2 mm. hole between the tube compartment and the base, to allow free passage of air avoiding a build up in pressure in the collection compartment.

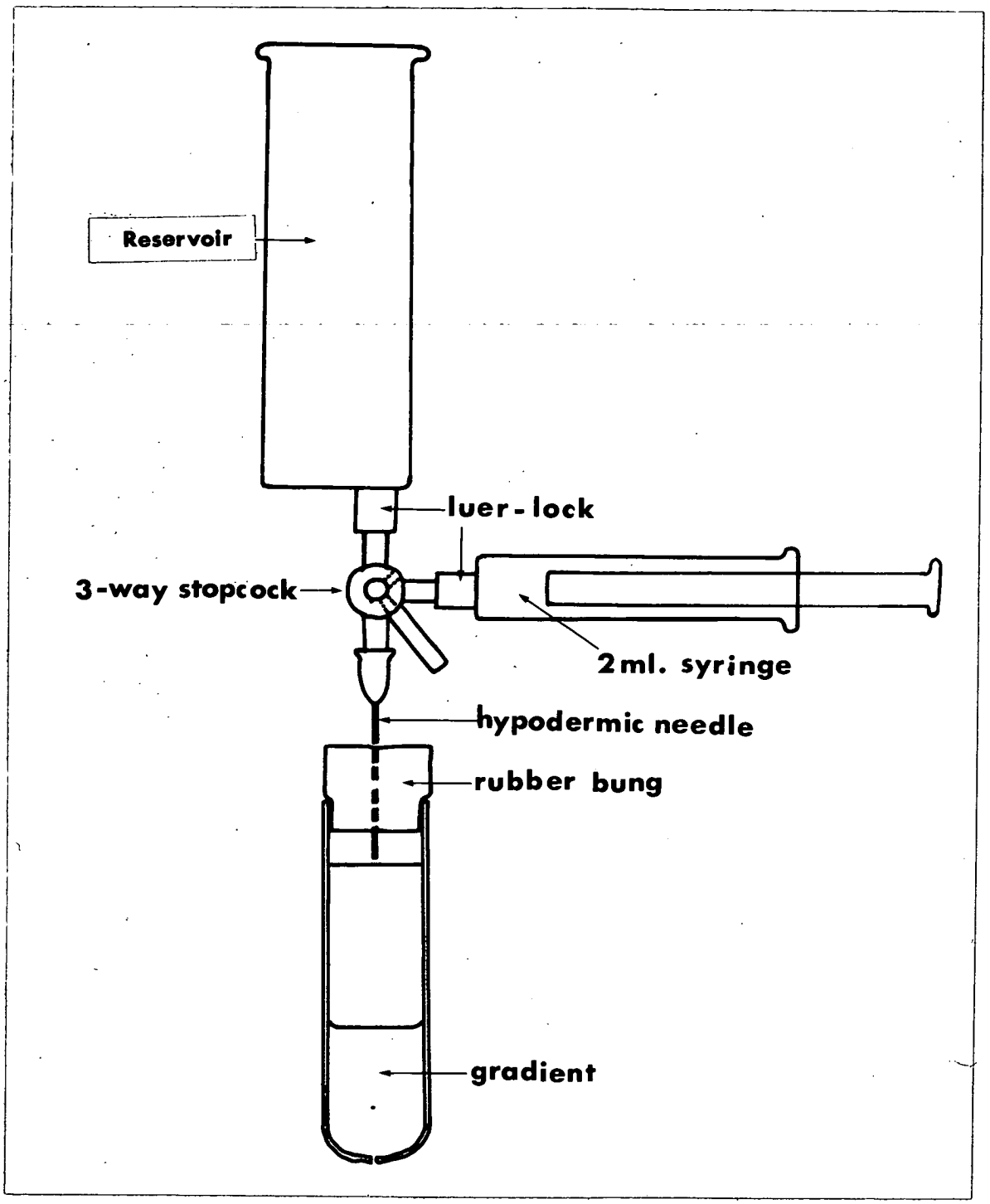
Figure 39. The Eaton Press.


7.6cm.
2cm.
Cylinder
Block.
8
10 cm.
REDUCED DIAMETER
1mm
8
10cm.
1.95cm.
Piston.
DEPRESSION
GROOVE
Collecting
Tube
Block.
10cm.
2mm.
7.6cm.

Appendix 3

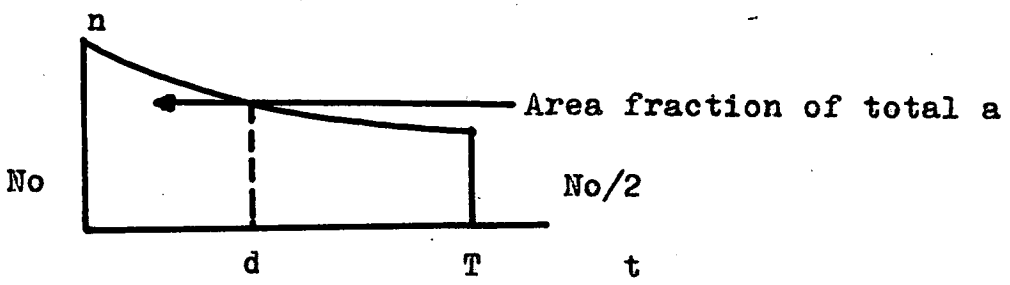
The Fractionating Device

Figure 40 shows a diagram of the device. Essentially it was composed of a 20 ml. syringe (without its piston), a 2 ml. syringe and a hypodermic needle all connected via a three way stop-cock. The 20 ml. syringe served as a reservoir for liquid paraffin and could be connected to the 2 ml. syringe by opening the tap between these two. Once the 2 ml. syringe was full the tap could be changed so that the small syringe was now open to the needle. The rubber bung over the needle was tightly inserted into the tube containing the gradient to be fractionated. The bottom of the tube was pierced with a fine needle and, because the seal between the tube and the bung was airtight, little liquid was lost. Fractions of a given volume could then be collected by dispensing a given amount of liquid paraffin from the small syringe.


Reservoir
luer-lock
3-way stopcock
2ml. syringe
hypodermic needle
rubber bung
gradient

Appendix 4

Derivation of the Equation Relating Stage in the Cell Cycle to the Number of Cells that have passed that Point



$n = Noe^{-kt}$ when $t = 0$ $n = 0$

and when $t = T$ $n = \frac{No}{2}$

$= Noe^{-kt}$

$e^{-kt} = \frac{1}{2}$

$kT = -\log_e \frac{1}{2}$

find d, such that

$$\int_0^d Noe^{-kt}dt = a\int_0^T Noe^{-kt}dt$$

$$(e^{-kt})_0^d = a\,(e^{-kt})_0^T$$

$$e^{-kd} - 1 = a\,e^{-kt} - a$$

$$e^{-kd} = 1 - \frac{a}{2}$$

$$kd = -\log_e(1 - \frac{a}{2})$$

$$\frac{d}{T} = \frac{\log_e(1 - \frac{a}{2})}{\log_e \frac{1}{2}}$$

This equation was derived by Dr. M.H. Williamson in 1964 when he held a lectureship in the Department of Zoology, University of Edinburgh.

Appendix 5

Index to Materials and Methods

Appendix 6

SYNTHESIS OF ENZYMES AND DNA IN SYNCHRONOUS CULTURES OF *SCHIZOSACCHAROMYCES POMBE*

By

C. J. BOSTOCK, Dr. W. D. DONACHIE

Dr. MILLICENT MASTERS

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(*Reprinted from Nature, Vol.* 210, *No.* 5038, *pp.* 808–810, *May* 21, 1966)

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SYNTHESIS OF ENZYMES AND DNA IN SYNCHRONOUS CULTURES OF *Schizosaccharomyces pombe*

By C. J. BOSTOCK, DR W. D. DONACHIE*, DR. MILLICENT MASTERS*† and PROF. J. M. MITCHISON

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THERE has been considerable interest in recent years in the production of enzymes by synchronous cell cultures[1–13]. The long-term aim of this work is to understand the mechanisms which regulate the rate of synthesis of specific proteins during the cell cycle. We have assayed a number of enzymes in synchronous cultures of the fission yeast, *Schizosaccharomyces pombe*. We have also used these cultures to determine the pattern of DNA synthesis. This has previously been an intractable problem because of the difficulty of selectively labelling the DNA[14].

Synchronous cultures of 200–700 ml. of *S. pombe* (N.C.Y.C. 132) in Edinburgh minimal medium[15] ($1 - 4 \times 10^6$ cells/ml.) were prepared by the method of Mitchison and Vincent[16]. Glucose gradients were used instead of sucrose gradients in order to avoid perturbing the culture by changing the carbon source. In some cases, an asynchronous control culture was prepared from the remaining cells in the gradient after the top layer had been removed to make the synchronous culture. The cultures were grown at 32° C and samples removed at intervals for enzyme assay (washed and freeze-dried), DNA assay, scoring of cell plates and, sometimes, cell numbers. Aspartate transcarbamylase (ATCase) was assayed by resuspending freeze-dried samples in 0·1 ml. 1 M glycine (*p*H 9·5) and 0·1 ml. 0·25 M L-aspartic acid (*p*H 9·5) in an ice bath and then adding 0·1 ml. carbamyl phosphate (4 mg/ml.) to complete the reaction mixture. The reaction was carried out at 28° C and the carbamyl aspartic acid formed estimated by the method of Gerhart and Pardee[17]. Ornithine transcarbamylase (OTCase) was assayed in exactly the same way, except that 0·03 M L-ornithine was substituted for the aspartic acid. The citrulline formed was estimated by the same method as was used for carbamyl aspartic acid. Alkaline phosphatase

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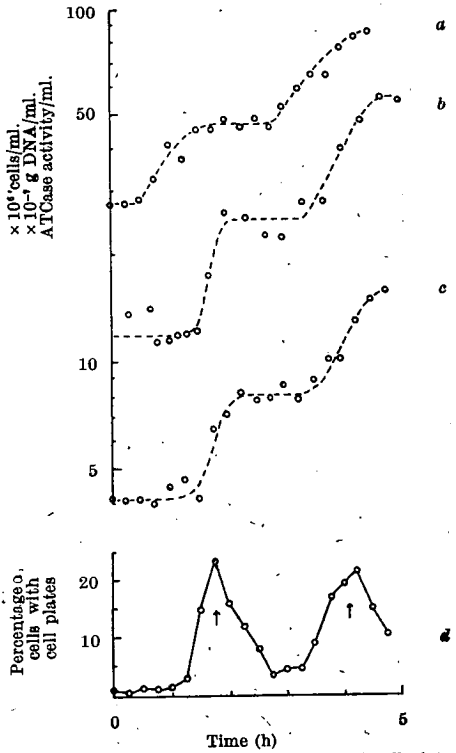

Fig. 1. ATCase (*a*), DNA (*b*), cell number (*c*) and cell plate index (*d*) in a synchronous culture

was assayed by the method of Torriani[18]. Sucrase (invertase) was assayed by the method of Masters *et al.*[2]. A similar method was used for maltase, with maltose as the substrate. DNA was measured by a modified version of the fluorometric technique of Kissane and Robins[19]. This is up to a hundred times more sensitive than the diphenylamine reaction[20]. Comparative tests with the two methods gave very similar DNA values with this material. The cell plate index (percentage of cells showing the transverse cell plate which precedes division) was measured on dried smears which were negatively stained with 10 per cent waterproof black drawing ink and then stained with 0·25 per cent crystal violet. This index is analogous to a mitotic index and is constant at about 10 per cent in a normal culture growing asynchronously. Cell numbers were determined by counting in a haemocytometer.

In the synchronous culture shown in Fig. 1, measurements were made of ATCase, DNA, cell numbers and cell

plate index. There were two synchronous divisions and, slightly before them, two peaks in the cell plate index. DNA synthesis was sharply periodic with a doubling in total amount at about the time that the cell numbers doubled. In a series of similar experiments, the mean time of DNA doubling was a little earlier (about 15 min) than the doubling in cell numbers. This relation is shown in Fig. 3*c*. The single nucleus of *S. pombe* divides shortly before the appearance of the cell plate[21]. It seems, then, that DNA synthesis, at least in these synchronous cultures, has the following pattern. At the completion of nuclear division, the DNA synthetic period (S) commences almost immediately. There is therefore little or no presynthetic ($G1$) period between nuclear division and the S period. It is difficult to determine the exact length of the S period but in a number of experiments (including that in Fig. 1) the rate of increase of DNA during the S period was the same as that of the cell numbers. This would imply a very fast replication process. Finally, there is a long postsynthetic period ($G2$) before the next nuclear division. A restricted period of DNA synthesis during the cell cycle is the usual pattern of higher cells and it also occurs in budding yeast[22,23]. It is less common for $G2$ to occupy most of the cycle, though two other organisms in which this is known to happen are the slime mould *Physarum*[24] and sea-urchin eggs[25]. DNA synthesis in *S. pombe* is at present being explored by one of us (C. J. B.).

Fig. 1 also shows that ATCase activity rose in steps. Assuming that the enzyme is stable, it is synthesized only during a restricted period of the cell cycle. The length of this period would seem to be rather longer than that for DNA synthesis. The time of initiation of the burst of synthesis is the middle of the cycle in each of the two cycles. A similar result with another synchronous culture is shown in Fig. 2. In this experiment, a control asynchronous culture was set up from the same gradient and it showed an exponential increase in ATCase activity without any steps. The periodic nature of enzyme synthesis is therefore a property of the cell cycle and not simply the consequence of re-inoculating the cells from the gradient into fresh medium. The initiation times from five experiments are shown on a cell cycle map[2,4,8] in Fig. 3*a*.

OTCase activity followed a very similar pattern except that the steps were less well defined and somewhat more variable in their timing in the cycle (Figs. 2 and 3*b*). The average initiation times for the ATCase and OTCase steps are plotted on a cell cycle map in Fig. 3*c*, together with the mean cell plate peak and the mean mid-point of DNA synthesis. Although there is a difference between the ATCase and OTCase means, it is not statistically significant.

We have also measured the activity of three other

enzymes—sucrase, maltase and alkaline phosphatase. The first two are at basal level in Edinburgh minimal medium (where the carbon source is glucose) but can be induced by the appropriate carbon source. Alkaline phosphatase appears to be constitutive since its activity per cell cannot be altered either by raising or by lowering the phosphate content of the medium. This enzyme has also

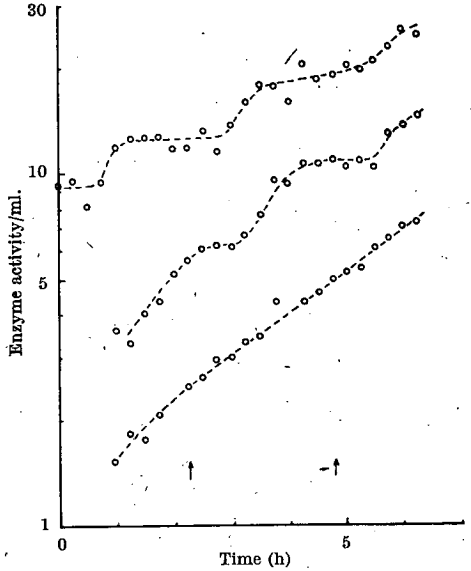

Fig. 2. OTCase (*a*) and ATCase (*b*) in a synchronous culture, and ATCase in a control (*c*) asynchronous culture from the same gradient. The arrows indicate the cell plate peaks in the synchronous culture, as in Fig. 1

been shown to be constitutive in another yeast[26]. With all three enzymes there was a continuous increase in activity through the cell cycle in synchronous cultures. Three experiments with synchronous and control cultures are shown in Fig. 4. There was an initial fluctuation in the maltase activity which occurred in both synchronous and control cultures and was probably a result of the synchronizing procedure. These enzymes, unlike ATCase and OTCase, appear to be synthesized continuously and smooth curves have been drawn provisionally through the points on the logarithmic plot in Fig. 4, but there may in fact be changes in the rate of synthesis similar to those found with repressed enzymes in synchronous bacterial cultures (alkaline phosphatase in *Bacillus subtilis*[5], alkaline phosphatase and β-galactosidase in *Escherichia coli*[3]). This point is being investigated by one of us (J. M. M.).

One purpose of this work was to discover whether all enzyme synthesis during the cell cycle was periodic, as reported for *Saccharomyces cerevisiae*[8,9], or whether it was

periodic for some enzymes and continuous for others, as in bacteria[3,5,6]. The results reported here indicate that both periodic and continuous syntheses of enzymes occur. Furthermore, it was found that basal synthesis of the sucrase and maltase was continuous, while there was periodic synthesis of ATCase and OTCase which may be controlled autogenously[3]. In this respect, therefore, the results obtained with *S. pombe* are identical to those obtained with *B. subtilis* and *E. coli*[2-6]. The constitutive synthesis of alkaline phosphatase was also continuous. An opposite result to this has been found in budding yeast where there are two steps in this enzyme in each cycle[8]. This may be due to differences in the degree of repression in the two systems since alkaline phosphatase activity in synchronized *B. subtilis* is continuous when fully repressed[5] and stepwise when derepressed[6]. The continuous synthesis of this enzyme and of the sucrase and maltase is in accordance with the hypothesis that periodic enzyme synthesis results from the operation of a feed-back of end-product on enzyme synthesis[3,4,6,27,28]. It is also possible to explain continuous synthesis in terms of periodic transcription through the cycle of a number of genes in different positions on the genome[9]. Without very accurate assays, it is impossible to distinguish a curve showing continuous rise in enzyme activity from one with more than two or three steps. This explanation would not, however, account easily for the doublings in rate of synthesis at a particular point in each cycle in the bacterial systems[2,5].

Another object of this work was to determine whether

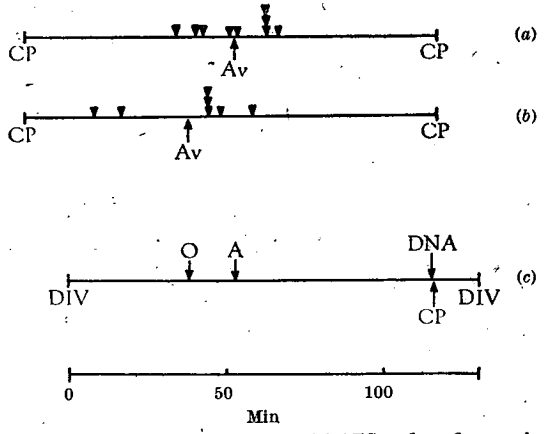

Fig. 3. Cell cycle maps of enzyme steps. (*a*) ATCase from five synchronous cultures. Each triangle is the initiation time. CP is the cell plate peak. (*b*) OTCase from four synchronous cultures. (*c*) Overall map for ATCase (A) and OTCase (O). DIV is cell division (mid-point of rise in cell numbers). DNA is the mid-point of rise in DNA

the initiation of periods of enzyme synthesis was closely connected with periods of gene replication. The results show particularly clearly that initiation of enzyme steps can occur during periods when there is no net DNA synthesis. This confirms for *S. pombe* the conclusion reached for *B. subtilis*[6].

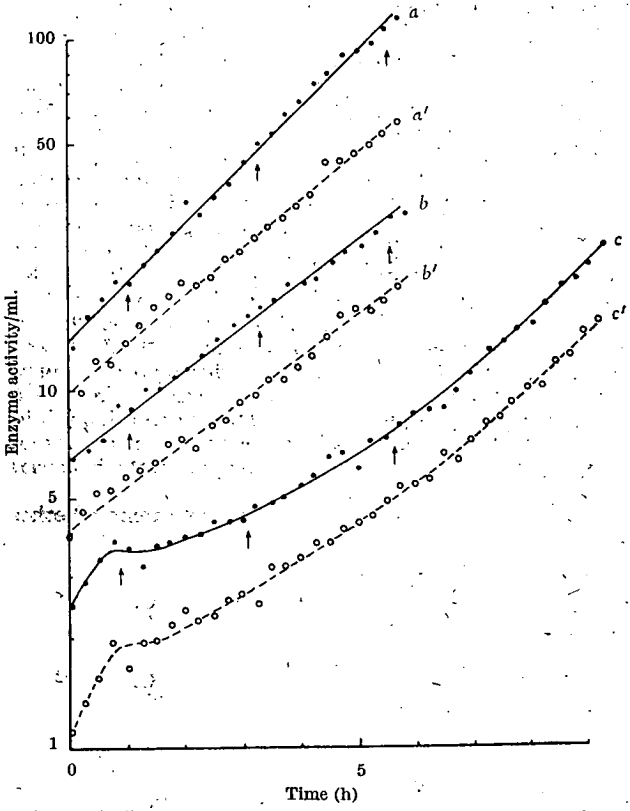

Fig. 4. Alkaline phosphatase (*a*), sucrase (*b*) and maltase (*c*) in synchronous cultures and in control (*a'*, *b'* and *c'* respectively) asynchronous cultures from the same gradients. The arrows indicate the cell plate peaks in the synchronous cultures, as in Fig. 1

These results therefore extend our knowledge of the cell cycle of fission yeast and indicate that similar modes of enzyme synthesis are found in both prokaryotic and eukaryotic cells.

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[1] Abbo, F. E., and Pardee, A. B., *Biochim. Biophys. Acta*, **39**, 478 (1960).
[2] Masters, M., Kuempel, P. L., and Pardee, A. B., *Biochem. Biophys. Res. Commun.*, **15**, 38 (1964).
[3] Kuempel, P. L., Masters, M., Pardee, A. B., *Biochem. Biophys. Res. Commun.* **18**, 858 (1965).
[4] Masters, M., and Pardee, A. B., *Proc. U.S. Nat. Acad. Sci.*, **54**, 64 (1965).
[5] Donachie, W. D., *Nature*, **205**, 1084 (1965).
[6] Masters, M., and Donachie, W. D., *Nature*, **209**, 476 (1966).
[7] Cummins, D. J., *Biochim. Biophys. Acta*, **85**, 341 (1965).
[8] Gorman, J., Tauro, P., LaBerge, M., and Halvorson, H., *Biochem. Biophys. Res. Commun.*, **15**, 43 (1964).
[9] Halvorson, H., Gorman, J., Tauro, P., Epstein, R., and LaBerge, M., *Fed. Proc.*, **23**, 1002 (1964).
[10] Sylvén, B., Tobias, C. A., Malmgren, M., Ottoson, R., and Thorell, B., *Exp. Cell Res.*, **16**, 75 (1959).
[11] Hotta, Y., and Stern, H., *Proc. U.S. Nat. Acad. Sci.*, **49**, 648 (1963).
[12] Hotta, Y., and Stern, H., *Proc. U.S. Nat. Acad. Sci.*, **49**, 861 (1963).
[13] Brent, T. P., Butler, J. A. V., and Crathorn, A. R., *Nature*, **207**, 176 (1965).
[14] Mitchison, J. M., *Symp. Intern. Soc. Cell Biol.*, **2**, 151 (1963).
[15] Mitchison, J. M., and Gross, P. R., *Exp. Cell Res.*, **37**, 259 (1965).
[16] Mitchison, J. M., and Vincent, W. S., *Nature*, **205**, 987 (1965).
[17] Gerhart, J. C., and Pardee, A. B., *J. Biol. Chem.*, **237**, 891 (1962).
[18] Torriani, A., *Biochim. Biophys. Acta*, **38**, 460 (1960).
[19] Kissane, J. M., and Robins, E., *J. Biol. Chem.*, **233**, 184 (1958).
[20] Burton, K., *Biochem. J.*, **62**, 315 (1956).
[21] Mitchison, J. M., *Exp. Cell Res.*, **13**, 244 (1957).
[22] Williamson, D. H., and Scopes, A. W., *Exp. Cell Res.*, **20**, 338 (1960).
[23] Williamson, D. H., *J. Cell Biol.*, **25**, 517 (1965).
[24] Nygaard, O. F., Güttes, S., and Rusch, H. P., *Biochim. Biophys. Acta*, **38**, 298 (1960).
[25] Hinegardner, R. T., Rao, B., and Feldman, D. E., *Exp. Cell Res.*, **36**, 53 (1964).
[26] Weimberg, R., and Orton, W. L., *J. Bact.*, **86**, 805 (1963).
[27] Pardee, A. B., in *Metabolic Control Colloquium of the Johnson Research Foundation* (Academic Press, in the press).
[28] Goodwin, B. C., *Nature*, **209**, 479 (1966).

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