PROTEIN SYNTHESIS DURING THE CELL CYCLE OF *ESCHERICHIA COLI*

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ABSTRACT

A method for studying the metabolically unperturbed bacterial cell cycle is presented and evaluated. The method allowed isotope incorporation and enzyme inductions to be performed on the exponential population in balanced growth. The cells were then killed, and resolved into size classes. These could then be analysed, and the data thus related to the original, unperturbed, exponentially growing cells.

Separation of cells into size classes was achieved by sedimentation velocity centrifugation, through a sucrose gradient, in an MSE, A-type, zonal rotor.

The efficiency of cell separation was assessed, and the correlation between cell size and cell age investigated. Mean cell length, mean cell volume, cellular protein and the rate of protein synthesis, were found to increase continuously across the rotor.

The changes in rates of synthesis of DNA, β-galactosidase and tryptophanase were found to occur at the expected times in the cell cycle, and compared well with similar studies upon synchronous cultures.

Small cells, selected from a zonal fractionation of live exponential cells, grew and divided synchronously. The rate of synthesis of RNA polymerase was studied in such synchronous cultures. The enzyme was found to be synthesised at all times during the cell cycle. There was no clearly discernible effect of gene dosage upon the synthesis of the enzyme.

The synthesis of membrane proteins was studied in fractions from the zonal rotor. Previous reports have suggested that certain proteins appear periodically in the membrane, only at, or near, the time of cell division. Such findings were not confirmed in these experiments.
Analysis of total cell protein synthesis by two-dimensional polyacrylamide gel electrophoresis also failed to reveal any proteins that were synthesised periodically during the bacterial cell cycle.

These findings are considered in relation to models which relate protein synthesis and cell division.
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INTRODUCTION

1 Protein Synthesis during the Cell Cycle:
   a) Enzyme Synthesis during the Cell Cycle:

   Much of our basic knowledge concerning the control of gene expression during the cell cycle derives from the study of enzyme synthesis during synchronous growth of bacterial and yeast cells. Certain enzymes, for which simple assay procedures exist, are particularly amenable to study. Enzyme activity, rather than enzyme protein, has been measured in all the experiments described below.

   Ample reviews of this field are available for prokaryotic and eukaryotic organisms (Donachie and Masters, 1969; Mitchison, 1969a; Mitchison, 1971; Halvorson, Carter and Tauro, 1971a). Only bacterial and yeast cell cycles are discussed below.

   The relationship between gene duplication and gene expression, during the cell cycle, has been studied under a variety of conditions. Figure 1 illustrates some idealised curves displaying patterns of enzyme synthesis under these various conditions.

   Some of the clearest data has emerged from studies of enzyme "potential". Kuempel et al (Kuempel, Masters and Pardee, 1965) have defined this as "the maximum ability for enzyme synthesis, such as should be obtained by complete derepression". Experimentally, aliquots were withdrawn from a synchronous culture and challenged with an inducer of enzyme synthesis. The amount of enzyme accumulated over a short time interval, as measured by its activity, provides a measure of the rate of enzyme synthesis. Characteristically, this rate is constant except for an abrupt rate doubling at a discrete time in the cell cycle (Figure 1b). These doublings of enzyme potential have been demonstrated in E. coli K12 for Aspartate transcarbamylase (ATCase), Alkaline phosphatase and Tryptophanase (Kuempel et al, 1965): in E. coli K12 for Tryptophanase,
FIGURE 1. Patterns of enzyme synthesis in a synchronous culture: duplication of the structural gene occurs mid-way through the cell division cycle.

A) Enzyme accumulates continuously, in either an exponential manner (dotted line), or a linear manner with a rate doubling (unbroken line). There is a maximum difference of only 3% between these two curves (Mitchison, 1971).

B) The rate of enzyme synthesis, or "potential" for enzyme synthesis. The corollary of A.

C) The discontinuous, periodic or stepwise mode of enzyme synthesis. Gene expression is unrestricted, but modulated by feedback repression - the Oscillatory Repression model: or gene expression is restricted to the time in the cycle when the gene is transcribed - the Linear Reading model.

D) The rate of enzyme synthesis predicted by the Linear Reading model, but so far never observed.

The first broad conclusion from such experiments was that at all times during the cell cycle genes were available for transcription. In other words, the potential for enzyme synthesis was unrestricted.

Secondly, the number of potential doublings correlated with the expected number of gene doublings. In all cases this was one. However, two doublings of β-Galactosidase potential were observed in an F′lac+ lac+ diploid (Donachie and Masters, 1966).

Thirdly, doublings in enzyme potential did not occur in the absence of Deoxyribonucleic acid (DNA) synthesis. This was shown for D-Serine deaminase in thymine starved cells of E. coli 15T (Donachie and Masters, 1966) and for β-Galactosidase in fluorodeoxyuridine treated E. coli B/r (Pato and Glaser, 1968).

Lastly, attempts were made to precisely correlate the doubling of enzyme potential with the duplication of the corresponding structural gene. Masters and Pardee (Masters and Pardee, 1965) demonstrated this for B. subtilis W23, in which the sucrase potential doubled at the same time as the sucrase transforming ability of the synchronous population. Helmsatter (Helmsatter, 1968) and Pato and Glaser (Pato and Glaser, 1968)
have shown that the temporal order of potential doublings corresponded to the temporal order of gene doublings, in experiments where the rate of DNA synthesis during the cell cycle was measured. However, in both cases the origin of DNA replication was assumed to be at about 60' on the E. coli chromosome. Replication was believed to proceed clockwise, unidirectionally from this origin. Duplication of the tryptophanase gene (tna, 73'), the β-Galactosidase gene (lac, 9') and the D-Serine deaminase gene (dsd, 45') would thus be expected to occur in succession. It was found that the potential doublings for these enzymes also occurred in that sequence. Our current knowledge of the origin and direction of DNA replication (Masters and Broda, 1971; Bird, Louarn, Martuscelli and Caro, 1972; McKenna and Masters, 1972; Hohlfeld and Vielmetter, 1973; Jonasson, 1973; Louarn, Funderburgh and Bird, 1974; Masters, 1975), would not profoundly alter the previously drawn conclusions, except for those relating to D-Serine deaminase. Assuming that the replicative terminus is diametrically opposite the origin; that the origin is at 71-73' on the E. coli chromosome (Masters, 1975); and that the velocity of replication forks is identical in either replicating arm of the chromosome, then the lac and dsd genes would be duplicated simultaneously. This expectation is demonstrated in this thesis.

In other types of experiment, rate measurements have not been made directly. Instead, the synchronous cells were grown in the presence of an inducer or repressor of enzyme synthesis. Under such conditions of optimal or basal gene expression, it was found that enzyme accumulated in a continuous linear manner, and that this rate of accumulation doubled abruptly at one specific point in the cell cycle (Figure 1a). This has been shown for fully repressed Alkaline phosphatase in E. coli K12 (Kuempel, Masters and Pardee, 1965) and in B. subtilis W23 (Donachie, 1965), and for fully induced β-galactosidase in E. coli B/r
Similar results have been obtained for Alkaline phosphatase, Acid phosphatase and Sucrase in the fission yeast, Schizosaccharomyces pombe, (Mitchison and Creanor, 1969). Fifteen further enzymes studied in S. pombe have been shown to accumulate in a continuous exponential manner (Figure 1a) through the cell cycle, (Mitchison, 1976).

These observations reinforce the conclusions relating to enzyme potential, although the rate doublings (where observed) have not been strictly correlated with gene doublings.

Having thus established that gene expression was unrestricted during the cell cycle, certain data, seemingly, was in contradiction to this finding.

Endogenous enzyme activity was measured in aliquots of synchronous cultures. The cells were not induced or repressed for the enzymes under study. Under these conditions, certain enzymes were found to accumulate in a discontinuous, stepwise, manner during the cell cycle, (Fig. 1c). This mode of enzyme synthesis has been observed in E. coli K12 for the enzymes Aspartate transcarbamylase, Dihydroorotase and Histidinol dehydrogenase (Kuempel, Masters and Pardee, 1965); in E. subtilis W23 for ATCase (Masters and Pardee, 1965; Donachie, 1965; Kuempel, Masters and Pardee, 1965), Ornithine transcarbamylase (OTCase) (Masters and Pardee, 1965; Donachie, 1965), Dehydroquinase and Histidase (Masters and Pardee, 1965), and Sucrase (Masters and Donachie, 1966). Discontinuous enzyme synthesis has also been observed during the cell cycle of Rhodopseudomonas spheroides for the following enzymes:- Succinyl Coenzyme A thiokinase, Aminolevulinic acid synthetase, Aminolevulinic acid dehydrase, Alkaline phosphatase and OTCase (Ferretti and Gray, 1968).
Two models have been proposed to explain these observations, neither of which are mutually exclusive.

Donachie (Donachie, 1965) has suggested that the in vivo instability of certain enzymes may produce patterns of enzyme synthesis which are apparently discontinuous. He considers that there is a steady state level of enzyme, created by a constant rate of synthesis being countered by a constant rate of inactivation. Duplication of the relevant structural gene doubles the rate of enzyme synthesis, and thus a new steady state level of enzyme is achieved.

The second model has been called the "Oscillatory Repression Model" (Mitchison, 1971), and has been developed in a number of papers (Kuempel, Masters and Pardee, 1965; Masters and Pardee, 1965; Masters and Donachie, 1966; Goodwin, 1966; Donachie and Masters, 1969; Mitchison, 1969a). This theory holds that the end product of a metabolic pathway can act as a co-repressor for the synthesis of the enzymes in that pathway. Consequently, the cellular level of enzyme would be auto-regulated by a negative feedback system which involves gene, messenger ribonucleic acid (mRNA), enzyme and end product in the control circuit. Such a system, it is claimed, would create its own, self-generating, oscillations, with a periodicity equal to the generation time of the cells under study (Goodwin, 1966).

However, other explanations for discontinuous enzyme synthesis have been proposed. Firstly, there is the very serious possibility that the observed patterns of enzyme synthesis were a consequence of a metabolic perturbation produced by the synchronising procedure (Mitchison and Creanor, 1969; Mitchison, 1976). In this context, it is noteworthy that Bellino (Bellino, 1973), using a non-perturbing technique, (the membrane-elution method - Helmstetter, 1967), observed
a continuous exponential mode of synthesis for ATCase in *E. coli* B/r.

In the system used by Bellino, exponentially growing cells were bound to a membrane filter (Helmstetter, 1967), and the synthesis of ATCase was repressed by addition of exogenous uracil. The enzyme was then assayed in progeny cells eluted from the membrane. This approach required that ATCase synthesis was repressed immediately upon addition of exogenous uracil, and that the level of repressed enzyme did not significantly contribute to the endogenous level. Bellino manipulated the endogenous enzyme level by partially derepressing the genes of the pyrimidine pathway with 6-azauracil (Yates and Pardee, 1957; Hanschumacher and Pasternak, 1958; Beckwith, Pardee, Austrian and Jacob, 1962; O'Donovan and Neuhard, 1970). Arguably, this very procedure may well have altered the normal flux through the negative feedback control loop. This experiment alone is therefore not sufficient to discount the previously observed discontinuous enzyme syntheses.

However, Mitchison (Mitchison, 1976) has performed some carefully controlled experiments upon the *S. pombe* cell cycle, from which he also concluded that enzymes were synthesised continuously throughout the cycle. He also demonstrated that the synchronising procedure was sufficient to produce periodicities of enzyme synthesis in this organism. This was not so for all enzymes studied, nor is it certain how these observations relate to other organisms.

A further explanation for discontinuous enzyme synthesis arises from the work done with budding yeasts (Halvorson, Carter and Tauro, 1971a). Discontinuous syntheses have been observed for a large number of enzymes, which have been summarised and discussed in detail by Mitchison (Mitchison, 1969a; Mitchison, 1971). The proposed "Linear
"Linear Reading Model" suggests that transcription proceeds sequentially along the chromosome. Genes are thus expressed only at the time of their transcription, resulting in bursts of enzyme synthesis as each gene is expressed.

Such a model implicitly requires a short mRNA half-life. However, recent observations (Creanor, May and Mitchison, 1975; Fraser, 1975) in *S. pombe* have shown that some messenger half-lives may be as long as 40-50 minutes.

Enzyme stability and enzyme potential have rarely been studied in budding yeasts. Sebastian et al (Sebastian, Carter and Halvorson, 1973) have shown that OTAase and β-Galactosidase are both stable enzymes, and both exhibited an unrestricted potential for synthesis. The endogenous enzyme levels followed a discontinuous mode of synthesis during the cell cycle. Such findings are difficult to accommodate within the Linear Reading Model.

The opposing facets of the Linear Reading Model and the Oscillatory Repression Model have yet to be fully reconciled. However, they both provide models for gene expression which are relevant to a wider consideration of protein synthesis during the cell cycle.

b) **Protein Synthesis and Cell Division:**

The foregoing summary has considered the temporal control of gene expression during the cell cycle.

The biochemistry of cell division is ill understood, and even less is known of the temporal control of this biochemistry.

The work of Helmatetter and co-workers (Helmatetter and Cooper, 1968;
Cooper and Helmstetter, 1963; Pierucci and Helmstetter, 1969) has provided an outline description of the bacterial cell cycle. Their model represents a basis for a more detailed consideration of the temporal relationships within the cell cycle. Briefly, their model envisages a period of protein synthesis (I) necessary for the initiation of chromosome replication. (Simultaneous with this initiation, the next cycle of I is begun.) Chromosome replication proceeds for 40 minutes (C period), upon completion of which, cell division ensues after a further 20 minutes (D period). The cell cycle is 60 minutes long.

It has been shown that completion of a round of DNA replication is a prerequisite of cell division (Clark, 1968; Helmstetter and Pierucci, 1968). Other aspects of DNA metabolism - repair and recombination - are also involved in the regulation of cell division, (Slater and Schaechter, 1974; Gudas and Pardee, 1975). A full discussion of the role of DNA metabolism is not given here. The topic is considered in some detail elsewhere (Slater and Schaechter, 1974).

Also necessary for cell division is a period of protein synthesis. In synchronous cultures, inhibition of protein synthesis during the C period was found to abolish subsequent cell division (Pierucci and Helmstetter, 1969). During the D period, inhibition of protein synthesis had no effect upon division. If DNA replication was completed in the absence of protein synthesis, cell division did not occur until protein synthesis had been restored for a time which was inversely related to the extent of chromosome replication at the time of the inhibition. Pierucci and Helmstetter (Pierucci and Helmstetter, 1969) concluded that, concurrent with DNA replication was an equal period of protein synthesis, completion of both processes being required
for cell division.

*E. coli* B/r has been synchronised by heat shock (Smith and Pardee, 1970). Cells maintained at 45°C for 16 minutes and then returned to 37°C, divided synchronously. Incorporation of p-fluorophenylalanine into cellular protein increased the sensitivity to the heat shock treatment. It was concluded that the heat shock damaged a protein structure which was elaborated during the cell cycle as a preparation for cell division. The shock caused all the members of the asynchronous population to recommence simultaneously the elaboration of this structure, leading to the subsequent synchronous division of the population.

Jones and Donachie (Jones and Donachie, 1973) have confirmed the requirement of a similar period of protein synthesis in *E. coli* K12-7T. They have also demonstrated that, for cell division to occur, a brief transcriptional event, at or near termination of DNA replication, is required.

In all their experiments, cells were "pretreated" in order to synchronise rounds of chromosome replication within the population. The cells were first exposed to rifampicin, thus inhibiting RNA and protein synthesis. DNA replication proceeded to termination. Only cells which had already completed DNA replication divided in the presence of the antibiotic. Rifampicin was then removed from the medium as well as thymine. Protein synthesis and remaining cell division took place, although DNA replication was inhibited. Readdition of thymine allowed a synchronous reinitiation of a round of chromosome replication in every cell in the population, lasting about 40 minutes. Cell division followed 5-10 minutes after completion of the round of DNA replication.
If protein synthesis was inhibited during the synchronous round of chromosome replication, but allowed to resume before termination of replication, cell division followed normally. Jones and Donachie concluded that the period of protein synthesis which is normally concurrent with DNA synthesis, had occurred during the thymine starvation step of their "pretreatment". However, they found that inhibition of protein synthesis at termination of DNA replication did block subsequent cell division. It was concluded that protein synthesis was required at termination of replication, for cell division to ensue. The protein(s) synthesised at this time was (were) called TAP(s); Termination Associated Protein(s).

Jones (Jones, 1974) has reported the discovery of the putative termination proteins in the membrane fraction of E. coli K12 T7. He used a selective labelling regime in which the proteins of cells undergoing chromosome replication were labelled with $^{14}$C-leucine, whilst cells terminating replication were labelled with $^{3}$H-leucine. Jones mixed the separately labelled cells and analysed the membrane proteins by Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis, (SDS-PAGE). A unique, tritium labelled protein band of molecular weight c.60,000 (designated TAP 1) was found. Analysis of the inner membrane proteins only, revealed TAP 1 and also a second, tritium labelled, band of molecular weight c.20,000 (TAP 2).

In a similar experiment Jones postponed the tritium labelling until after completion of DNA replication. The analysis of the isotope distribution in the gels did not reveal any exclusively tritium labelled species. Jones inferred that TAP 1 and TAP 2 were synthesised (or inserted into the membrane) solely at termination of DNA replication.

These findings form the basis for a model of the cell division
cycle (Jones and Donachie, 1973; Donachie, Jones and Feather, 1973). Initiation of DNA synthesis occurs simultaneously with initiation of a period of protein synthesis. These two processes run concurrently for 40 minutes. Termination of DNA replication induces the synthesis of the termination proteins. Having thus fulfilled the three requirements for cell division, the cell divides (without further protein, DNA or RNA synthesis) some 20 minutes later. The events taking place in this 20 minutes are not known.

The control of cell division in a temperature sensitive mutant, ts 52, has been studied (Zusman, Inouye and Pardee, 1972). This mutant continued DNA and protein synthesis at the restrictive temperature, but cell division was blocked and the cells thus formed filaments. A synchronous division of these filaments occurred about 15 minutes after exposure to chloramphenicol, rifampicin, or transfer to minimal medium. These workers have proposed a model to account for their findings. A non-protein effector, $\beta$, is believed to be involved in the process of peptide bond formation. Normally $\beta$ is present in excess, but in the ts 52 mutant its production is reduced. The small amounts of $\beta$ are swept into other metabolism, and it thus only accumulates when peptide bond formation is inhibited or reduced. It is proposed that $\beta$ interacts with $\alpha$ - a protein synthesised only at termination of DNA replication. The $\alpha\beta$ complex initiates cell division at the division site.

Inouye (Inouye, 1969) has studied division in the ts 27 mutant. Although DNA replication was blocked at the restrictive temperature, division of the bacterial cell continued. Inouye proposed that a factor, M, possibly attached to an "apparatus" which co-ordinates
replication and septum formation, normally blocks cell division. It was further assumed that termination of DNA replication causes synthesis of an unstable factor, I, which relieves the block established by M. Division thus ensues. The mutant is supposed to have a defective M factor, which reversibly dissociates from the "apparatus" so that septum formation occurs without completion of DNA synthesis.

There is little hard evidence for the existence of these factors, but it is noteworthy that both models demand the periodic production of a protein species at one discrete time in the cell cycle.

Inouye and Pardee (Inouye and Pardee, 1970) studied labelled membrane proteins of E. coli K12 in much the same way as Jones (Jones, 1974). They separated cells into age classes by sedimentation velocity centrifugation of the parent population. (This technique is discussed more fully below). They then compared membranes from young and old cells, which had been labelled with different isotopes and analysed by SDS-Polyacrylamide gel electrophoresis. Interestingly, these authors could detect no protein species synthesised only at one time in the cell cycle.

Churchyard and Holland (Churchward and Holland, 1976) claim to have found a membrane protein of molecular weight 76,000, which appears only at the time of cell division. This, and the TAPs of Jones (Jones, 1974), are some of the very few reports claiming to have identified periodically synthesised proteins in bacteria, ostensibly concerned with the process of cell division. Considerable caution must be used in the interpretation of such findings. Jones studied cells which had been subjected to cycles of metabolic block. Churchward and Holland, although studying a less perturbed synchronous population, failed to adequately resolve the many bacterial membrane
proteins on their SDS-Polyacrylamide gels. Furthermore, certain reported differences in the membrane proteins of wild-type and dnaB cells of E. coli (Shapiro et al, 1970) were later shown to depend on the conditions used to disaggregate the membranes in SDS prior to electrophoresis (Shapiro et al, 1971).

It is therefore important to establish that apparently periodically synthesised proteins do not arise as an artefact of the synchronising procedure, or of the SDS-Polyacrylamide gel system.

Dix and Helmatetter (Dix and Helmatetter, 1973) have studied the relationship between termination of DNA replication and cell division in the strain of E. coli B/r also studied in this thesis. They confirmed that DNA and protein synthesis were required for division, but that once completed, division was resistant to inhibitors of these processes.

They also studied how synchronously growing cells proceeded towards the potential to divide in the presence of chloramphenicol. Once the chloramphenicol block was relieved, inhibitors of DNA synthesis were added. They concluded that DNA replication could be completed in the absence of protein synthesis, but that additional protein or RNA synthesis was required for "processing" the chromosomal structure to a form necessary for division. This "processing" was believed to be inhibited by inhibitors of DNA synthesis. This view is perhaps untenable in the light of evidence produced elsewhere (Maranouchi and Messer, 1973). It was shown that DNA replication was not completed in the absence of protein synthesis, a terminal segment remaining unreplicated. Resumption of protein synthesis in the presence of inhibitors of DNA synthesis would therefore not permit cell division
since termination of DNA replication could not take place.

However, the requirement of a terminal transcriptional event, for cell division, in this strain of *E. coli B/r*, has been clearly demonstrated (Dix and Helmstetter, 1973). They thymine starved exponentially growing cells, thus inhibiting DNA replication and allowing only cells with completed chromosomes to divide. Protein synthesis continued during the thymine starvation. Readdition of thymine allowed DNA replication to complete, followed by cell division some 15 minutes later. Addition of rifampicin, simultaneously with the thymine, abolished the subsequent division, indicating that transcription, at or near termination of replication, was required for cell division. This finding is considered in this thesis in view of the data and models presented above.

2 Methods used in the study of the Cell Cycle:

a Introduction:

Nearly all the experiments described in the previous sections have been performed on synchronously growing cells. It must be constantly borne in mind that the very act of producing synchronous growth in a previously asynchronous population is likely to perturb cellular metabolism. Some methods produce greater perturbations than others; some cellular processes are more susceptible to perturbation than others.

It is always desirable, where possible, to study synchronous cultures produced by more than one method.

Few techniques are available which can be fairly claimed not to perturb cell physiology. One particular method (zonal fractionation of whole populations of cells), successfully used for budding yeasts,
is developed for *E. coli* B/r in this thesis.

The merits and demerits of other systems are discussed below.

b) **Induction Synchrony:**

i) **Inhibitor Block:**

This procedure aligns cells at one stage in the cell cycle by means of a specific inhibitor; relieving the inhibition allows the cells to grow and divide synchronously. It is convenient in use, and is capable of providing large numbers of synchronised cells for study. However, it is a procedure which synchronises only the function specifically inhibited, or other causally related functions. Certain other aspects of cell physiology may not be synchronised by such treatment.

*Schizosaccharomyces pombe* has been synchronised in this way, (Mitchison and Creanor, 1971). DNA synthesis was inhibited for three hours with 2mM deoxyadenosine. Following its removal from the growth medium, there was a burst of synchronous DNA synthesis followed by a division. The second division was less synchronous and the time between divisions was only 60% of the normal generation time. Thus the "DNA-Division Cycle" was synchronised in this way. However, during the inhibitor block, protein and RNA synthesis continued, and so the resulting synchronous population contained abnormally large cells. Mitchison (Mitchison, 1971) has suggested that the "Growth Cycle" may thus become dissociated from the "DNA-Division" cycle.

Accordingly some replication related processes were studied (Sissons, Mitchison and Creanor, 1973). It was found that the potential for sucrase and maltase synthesis doubled during the cell cycle, as had been shown previously (Mitchison and Creanor, 1969) in synchronous
cultures produced by a selection method (Mitchison and Vincent, 1965). However, the discontinuous synthesis of ornithine transcarbamylase and aspartate transcarbamylase, previously observed in selection synchronies (Bostock, Donachie, Masters and Mitchison, 1966) was not observed in these induction synchronies. According to the model above, these syntheses are part of the "Growth Cycle" which had not been synchronised by the inhibitor block.

Perhaps this model of two cycles is a little speculative, but it is useful to consider the findings of Jones and Donachie (Jones, 1944; Jones and Donachie, 1973) in this light. It will be recalled that Jones synchronised DNA replication by means of a double inhibitor block. Following an inhibition of protein synthesis, DNA synthesis was inhibited. During this latter inhibition, protein synthesis was allowed to proceed and the cells would have become abnormally large. Relieving the DNA block allowed one synchronous round of replication followed by a synchronous cell division. A second cycle was not studied.

Arguably, in studying events causally related to the termination of DNA replication, Jones and Donachie were studying an aspect of the synchronised DNA-Division cycle. However, the synthesis, or assembly, of membrane proteins could well be part of the, perhaps unsynchronised, Growth Cycle. Clearly the findings of Jones (Jones, 1974) need to be reinvestigated using an alternative method.

ii) Starvation and Growth:

E. coli and Proteus vulgaris have been synchronised by diluting cells in early stationary phase into fresh growth medium (Cutler and Evans, 1966). These workers routinely found three or more surprisingly well phased synchronous divisions.
This method has been used with *Bacillus subtilis* W23 (Masters and Pardee, 1965; Donachie, 1965; Masters, Kuepel and Pardee, 1964; Masters and Donachie, 1966), in the study of enzyme synthesis during the cell cycle. It is difficult to assess the results because they have not been duplicated in synchronous cultures produced by alternative methods. However they are consistent with the general theories of prokaryotic enzyme synthesis described above. Unfortunately it is not possible to strictly exclude the criticism that the observed discontinuous patterns of enzyme synthesis arose from perturbations introduced by the synchronisation procedure.

Williamson and Scopes (Williamson and Scopes, 1960) used an elaborate and lengthy procedure to synchronise *Saccharomyces cerevisiae*. A stationary phase culture was treated with alternating cycles of cold growth medium and warm starvation medium, accompanied by decanting-off of small cells. The resulting homogeneous population was used as an inoculum to produce a synchronous culture.

Gorman et al (Gorman, Tauro, LaBerge and Halvorson, 1964) used a modification of this procedure in their studies of enzyme activity during the yeast cell cycle. They found that α-glucosidase, invertase and alkaline phosphatase were synthesised discontinuously during the cycle. Tauro and Halvorson (Tauro and Halvorson, 1966) have confirmed these findings using both the above method of synchronisation and the gradient selection method (Mitchison and Vincent, 1965).

c) **Selection Synchrony:**

i) **Introduction:**

This procedure involves selection of a discrete size class (usually the smallest) of cells from an asynchronous population.
These small cells are subcultured and divide synchronously for at least two generations. Since only a small percentage of the original asynchronous population is selected, the method produces less cells for study than the induction methods.

ii) Filtration:

The earliest attempts at selection were rather inelegant and often introduced quite serious disruptions of cellular physiology.

Abbo and Pardee (Abbo and Pardee, 1960) passed an asynchronous population of *E. coli* B through 24 layers of filter paper. The eluate, containing small cells, produced a well phased synchronously dividing population. However, both the rate of DNA and β-Galactosidase synthesis increased continuously in an exponential manner throughout the division cycle. Presumably, although division was synchronised, DNA replication was not.

A similar result was found by Nishi and Horiuchi (Nishi and Horiuchi, 1966) using the same selection method. They studied the potential for D-Serine dehydratase and β-Galactosidase synthesis in various strains of *E. coli* K12 during synchronous growth. In an F<sup>+</sup>lac strain, potential for both enzymes increased continuously, whereas in an Hfr strain the usual stepwise doubling of potential was seen. Nishi and Horiuchi concluded that F<sup>−</sup> strains could initiate DNA replication anywhere upon the chromosome, whereas Hfr strains initiated replication at the site of insertion of the F factor. With hindsight it seems more probable that these findings arose from the dissociation of replication from division in one experiment but not in the other.

Masters et al (Masters, Kuempel and Pardee, 1964) synchronised
E. coli K12 Hfr by this filtration method, and studied ATCase and β-Galactosidase potential. They found that ATCase potential doubled during the cell cycle, but that β-Galactosidase potential increased continuously. Presumably DNA replication had been synchronised in this experiment, but β-Galactosidase was perturbed whilst ATCase was not.

iii) Gradient Selection:

Mitchison and Vincent (Mitchison and Vincent, 1965) have described a convenient selection method which is applicable to several organisms. They collected and concentrated exponentially growing cells, and then layered the dense cell suspension upon a linear sucrose gradient in a centrifuge tube. Centrifugation through the gradient separated cells into size classes. The smallest cells were selected from the top of the gradient and were subcultured to produce a synchronously dividing population. The method has been used successfully with E. coli, Schizosaccharomyces pombe and Saccharomyces cerevisiae.

This procedure has been used to examine the potential for β-Galactosidase, tryptophanase and D-Serine deaminase synthesis in E. coli K12 (Donachie and Masters, 1966). The potential for each enzyme doubled at a specific point in the cell cycle, in both Hfr and F⁻ strains.

Convenient "shaken gradient" controls are easily applicable to this procedure. For example, in their studies of Schizosaccharomyces pombe, Bostock et al (Bostock, Donachie, Masters and Mitchison, 1966), found that ATCase and OTCase were synthesised discontinuously during the cell cycle. In a control experiment, after the gradient separation,
the gradient was mixed by shaking and an inoculum taken from this was subcultured. The cells divided asynchronously. ATCase and OTCase activity increased continuously in the growing culture, indicating that the gradient selection had not perturbed the synthesis of these enzymes.

Mitchison (Mitchison, 1976) has paid particular attention to this type of control experiment. He has shown that certain enzymes show virtually no perturbation of their synthesis after the gradient selection. Certain others show an initial perturbation which is usually corrected within the first generation. However, some enzymes exhibit a prolonged perturbation in their synthesis. A discontinuous mode of synthesis for three enzymes was observed over two generations even in the asynchronous controls. Mitchison concluded that nearly all the enzymes so studied in *Schizosaccharomyces pombe* are synthesised continuously during the cell cycle. Only one genuinely discontinuously synthesised enzyme was discovered, and that was Thymidinemonophosphate (TMP) Kinase.

The *Saccharomyces cerevisiae* cell cycle has been studied using the gradient selection method (Tauro, Halvorson and Epstein, 1968; Cox and Gilbert, 1970; Halvorson, Carter and Tauro, 1971b). The predictions of the linear reading model were borne out for a variety of enzymes. No asynchronous controls were carried out.

iv) Membrane Elution:

A very powerful technique for studying the bacterial cell cycle has been developed (Helmstetter and Cummings, 1963; 1964). Exponentially growing cells were bound to a millipore filter, which was then inverted, and new born cells eluted from the surface of the filter. Successive
fractions of the eluate were studied directly (Helmstetter, 1967; Helmstetter and Cooper, 1968), or alternatively young cells were collected and used to establish synchronous cultures. Both methods have been used to study the potential for synthesis of β-Galactosidase, tryptophanase and D-Serine deaminase (Pato and Glaser, 1968); the results from either approach were identical.

The method has proved to be extremely powerful in the analysis of DNA replication during the bacterial cell cycle (Helmstetter, 1967; Helmstetter and Cooper, 1968; Cooper and Helmstetter, 1968).

The analysis of the potential for enzyme synthesis during the cell cycle (Helmstetter, 1968; Pato and Glaser, 1968) has produced unexpected results for D-Serine deaminase potential, as detailed above.

The analysis of endogenous ATCase activity during the cell cycle (Bellino, 1973) has suggested that the enzyme is synthesised continuously rather than discontinuously. Clearly this isolated finding requires further investigation.

Unless specifically adapted, (Cummings, 1965; 1970) the method is limited to small numbers of cells and to one bacterial strain (E. coli B/r ATCC 12407).

d) Zonal Fractionation:

Conventional approaches to the cell cycle have involved manipulations (e.g. isotope incorporation, enzyme inductions etc.) upon synchronised, and possibly perturbed, populations.

Helmstetter (above) has carried out such manipulations on the asynchronous, steady state, unperturbed population. He then examined the progeny of the original population, thus permitting a retrospective analysis of its members.
Zonal separation of cells into size classes allows the asynchronous population to be directly examined. Following enzyme induction and/or isotope uptake, the population can be chilled, killed, collected, concentrated and finally separated into size classes. This separation is achieved by sedimentation velocity centrifugation through a suitable density gradient. Most commonly a sucrose gradient is used.

The technique is applicable to a wide variety of organisms and tissue culture cells (Mitchison, 1971), and is potentially capable of handling large numbers of cells.

The method assumes that, for example, enzyme activities do not change during the course of the size fractionation; or that the osmotic effect of the density gradient does not affect, for example, cell membrane proteins. It further assumes that separation is purely on a cell size basis, and that cell size and cell age are closely correlated (see Discussion).

Size fractionation of *E. coli* on sucrose gradients prepared in conventional centrifuge tubes has been only rarely used (Kubitschek, Bendigkeit and Loken, 1967; Manor and Haselkorn, 1967; Inouye and Pardee, 1970). The method resolves the bacteria poorly and is limited by the small numbers of cells which can be loaded upon a small tube gradient.

Anderson et al (Anderson, Barringer, Cho, Munley, Babelay, Canning and Rankin Jr., 1966) have pioneered the development of high capacity, low speed, zonal rotors. These cylindrical rotors can be pre-loaded, whilst spinning, with a large gradient (1300mls), and are capable of resolving more cells, more effectively than conventional tube gradients. The use of the zonal rotor is described
explicitly under Materials and Methods.

Zonal fractionation of asynchronous populations of *Saccharomyces cerevisiae* has been achieved with some substantial success (Sebastian, Carter and Halvorson, 1971). These workers have resolved budding yeasts into discrete, and equally homogeneous, size classes; mean cell volume more than doubled across the rotor. They have also shown that patterns of OTAase, α-glucosidase and β-galactosidase activity during the cell cycle, were identical in both synchronous cultures and zonal fractionated cells (Sebastian, Carter and Halvorson, 1971; 1973). Ribosomal RNA synthesis has also been studied in both zonal fractions and synchronous cultures, and again the data was almost identical in either experimental approach (Sogin, Carter and Halvorson, 1974).

Tan et al (Tan, Hartmann, Guntermann, Huttermann and Kuhlwein, 1974) have fractionated *Myxobacter* AL-1 using a Beckman Ti15 high speed zonal rotor. Their data is less clear than that obtained for budding yeasts, above. The synthesis of α-glucosidase during the *Myxobacter* cell cycle has been studied (Guntermann, Tan and Huttermann, 1975), and was found to oscillate during the cycle. No clear interpretation can be made of this isolated observation.

*E. coli* K12 has been fractionated using an A-type, low speed, zonal rotor (Beck and Park, 1976). The activity of three muerein hydrolases during the cell cycle of this strain was studied. No clear conclusions can be drawn from the data, since insufficient details of the efficiency of size and age resolution were given.

Much of this thesis is devoted to developing the technique of zonal fractionation of *E. coli* B/r, in an attempt to analyse temporal events in the unperturbed cell cycle.
MATERIALS AND METHODS

1 Bacterial Strains and Media:

The strain of *E. coli* used was supplied by C.E. Helmstetter - *E. coli B/r ATCC 12407*.

The afimbriate *E. coli B/r fim* was derived from a laboratory strain by K. Begg.

In all experiments the cells were grown in the minimal salts medium described by Helmstetter (Helmstetter, 1967), containing 0.2% glycerol as a carbon source.

2 Monitoring Cell Growth:

Cells were grown at 37°C on a New Brunswick gyrotatory shaker in a thermostatically controlled warm room.

Cell growth was monitored by measuring the optical density of the culture at 540nm. A Zeiss PMQII, or Hilger spectrophotometer (Hilger and Watts Ltd., London), was used.

Cell number was estimated using a Coulter electronic particle counter model A, equipped with a 30um orifice tube (Coulter Electronics, Great Missenden, Herts.). Cell samples were diluted for counting into a filtered electrolyte (0.9% NaCl, 0.05% NaN₃). Particle counts were maintained between 10,000 and 30,000, and background counts kept always below 500.

3 Cell Size Distributions:

In the absence of reliable electronic particle size measuring instruments (Harvey and Marr, 1966; Harvey, 1968; Kubitschek, 1969), photomicrography provided an accurate alternative (Teather, Collins and Donachie, 1974).
Fixed cells were immobilised on a thin film of 1.2% agar on a microscope slide. Cells were examined by phase contrast microscopy and photographed using a Zeiss Ultraphot microscope.

The film negatives were projected onto a screen using an enlarger, and the cell lengths measured manually. At least 200 cells were measured for each distribution.

Samples, similarly prepared for microscopy, were used for scoring the percent divided cells in the population.

4 Inhibition of Bacterial Growth:

Specific inhibitors of cell division, and of protein and DNA synthesis, were routinely used at the following final concentrations:

- Benzylpenicillin BP (sodium salt) (Solupen: Distal Products Ltd., Liverpool) at 20 or 60 units/ml (1665 units/mg).
- Chloramphenicol (Grade B, Calbiochem) at 200 μgm/ml
- Nalidixic Acid (Winthrop Laboratories, Newcastle) at 20 μgm/ml

Occasionally, sodium azide at 0.09% final concentration was used as a growth inhibitor.

A 0.4% formaldehyde solution, in the minimal medium, was used to store cells prior to photomicrography or cell number estimations. Cells thus fixed could be stored for several days at 4°C without loss of particle counts.

5 Radiisotope Incorporation and Counting Procedures:

The rate of DNA synthesis was measured by the incorporation of methyl 3H-thymidine (50 Ci/mmol: the Radiochemical Centre, Amersham),
into Trichloroacetic acid (TCA) precipitable material, following a short pulse (1-2 minutes). The radiochemical was added at 5 or 0.5 µCi/ml.

The rate of protein synthesis was measured by the incorporation of L-4,5-3H-leucine (43 Ci/mmol) into TCA precipitable material, following a short pulse (1-4 minutes). The radiochemical was added at 1 µCi/ml plus unlabelled L-leucine at 0.01-1.0 µg/ml.

Alternatively, 14C L-leucine (34.2 mCi/mmol) was used at 5 µCi/ml; or 35S-methionine (385 Ci/mmol) at 0.1-20 µCi/ml plus 0.05 µg of unlabelled methionine per ml, was used.

Where gel electrophoresis was to follow, labelling was always chased for 3 minutes with unlabelled amino acid at 200 µg/ml, so that only completed polypeptide chains were labelled.

Incorporation of isotope was terminated either by addition of cold 5% TCA containing 100 µg/ml of unlabelled precursor, or, where necessary, by addition of the relevant antibiotic.

For measuring incorporation of isotope, samples of labelled cells were applied to Whatman filter paper discs (2.4 cm diameter, Whatman). Up to 50 µls of cells were applied. The discs were washed twice with cold 5% TCA (containing excess of unlabelled precursor) for 30 minutes, and once in cold 80% ethanol. They were then dried and placed in 5 mls of a 0.5% solution of Butyl-PBD (2-(4'-tert-Butylphenyl)-5-(4''-Biphenyl)-1,3,4-Oxadiazole)(Ciba, Cambridge, England) in toluene, and counted in a Packard Tri-Carb liquid scintillation counter.

Larger volumes of labelled cells (0.1-1.0 mls) were precipitated with cold 5% TCA (containing excess unlabelled precursor) and collected by filtration on a 0.45 µm millipore filter. Precipitates were washed twice with cold 5% TCA and once with cold 80% ethanol. The filters
were then dried and counted as described above.

For counting mixtures of \( ^{14}\text{C} \) and \(^{3}\text{H} \) isotopes, the scintillation counter was adjusted such that less than 0.1\% of tritium spilled over into the \( ^{14}\text{C} \) channel. The spillover of \( ^{14}\text{C} \) into the tritium channel was 1\%. 

6 Macromolecular Estimations and Enzyme Assays:

a) Protein:

This was estimated in whole cells by the method of Lowry (Lowry et al., 1951).

b) β-Galactosidase:

Synthesis of the enzyme was induced with IPTG (Sigma: isopropyl-β-D-thiogalactopyranoside) at \( 10^{-2} \text{M} \) final concentration. The \( 10^{-1} \text{M} \) stock solution in water, was sterilised by autoclaving at 10 Pounds per square inch pressure for 10 minutes.

The enzyme was assayed essentially according to Loomis and Magasanik (Loomis and Magasanik, 1964).

Suitable aliquots of cells, diluted to 1 ml total volume, were added to a mixture of 0.8 mls of FM2 reducing buffer (below), 0.2 mls of Cetab (BDH, Poole, Dorset: Cetyl-trimethyl- ammonium bromide) at 1 mgm/ml, and 10 mls of a 1\% solution of sodium deoxycholate.

(The FM2 reducing buffer was prepared as follows:-
\[
\text{Na}_2\text{HPO}_4: 5.1 \text{ gms; NaH}_2\text{PO}_4: 16.8 \text{ gms; } \text{H}_2\text{O: 950 mls; } 10 \text{ mls of } 10^{-1} \text{M MgSO}_4: 2 \text{ mls of } 10^{-1} \text{M MnSO}_4: 6.8 \text{ mls of } \beta\text{-mercaptoethanol; plus water to a final volume of 1 litre.}
\]

Samples were kept on ice for at least 30 minutes, or overnight at \( 4^\circ\text{C} \), without loss of enzyme activity.
For assay, samples were preincubated at 28°C for 5 minutes, and the reaction commenced by timed additions of 0.6 mls of the substrate, ONPG (Sigma: o-nitrophenyl-β-D-galactopyranoside) prepared at M/75 in the minimal salts medium.

When sufficient yellow colour had developed the reaction was terminated with 1.3 mls of 1M Na₂CO₃, and the time of incubation noted. The colour was stable for several hours, or overnight at 4°C.

Cell debris was centrifuged off and the extinction at 420nm was measured. Enzyme units were expressed in terms of E₄₂₀/ml of cells/minute of incubation.

c) Tryptophanase:

The enzyme was induced with 1 mg/ml of L-tryptophan (Sigma).

The assay was performed according to Gartner and Riley (Gartner and Riley, 1965).

0.1 mls of cells, in a 0.1M potassium phosphate buffer, pH 7.0, containing 0.01M glutathione, were treated with a drop of toluene. Samples were retained on ice for no longer than three hours. Prior to assay, excess toluene was removed by blowing air gently over the surface of the samples. After preincubation (1-5 minutes) at 37°C, timed additions of 0.5 mls of prewarmed substrate were made to each sample. The substrate was a mixture of 0.5 mg/ml L-tryptophan and 55 μg/ml pyridoxal phosphate (Calbiochem, Grade A) in 0.1 M potassium phosphate buffer, pH 7.0. Incubation was continued for 30 minutes, and then terminated by addition of 2.3 mls of a solution of 4-dimethylaminocinnamaldehyde (BDM): the solution contained 233 mgs of reagent in 70 mls of 95% ethanol plus 10 mls of concentrated hydrochloric acid.
Maximum colour developed after 20-30 minutes and was stable for 1 hour, during which time the extinction at 625nm was measured against a no-cell blank.

Enzyme units were expressed in terms of $E_{625}/0.1 \text{ ml of cells/30 minutes of incubation.}$

d) **D-Serine Daminase:**

The enzyme was induced with 150 $\mu$g/ml of D-Serine. The assay was performed according to McFall (McFall, 1964).

0.4 ml of cells in 0.07M potassium phosphate buffer, pH 7.4, were toluenesed as described for the tryptophanase assay.

After preincubation of the samples at $37^\circ C$, 0.1 ml of D-serine substrate (10 mg/ml in buffer) was added, and incubation continued for 20 minutes.

The reaction was stopped by addition of 0.9 ml of a solution of 2-4-dinitrophenylhydrazine (0.17 mg/ml) in 1.2N HCl. The mixture was allowed to stand for a further 20 minutes at room temperature, and then 1.7 ml of 2.5N NaOH was added.

The extinction at 520nm was measured (within 30 minutes) against a no-cell blank.

7 **Cell Separation in the Zonal Rotor:**

a) **Introduction:**

Exponentially growing cells were resolved into size classes by sedimentation velocity centrifugation in a sucrose gradient performed in an A type, low speed, zonal rotor (MSE, London). The technique has been used for separation of bacterial (Beck and Park, 1976), yeast (Sebastian, Carter and Halvorson, 1971; 1973), and mammalian
Two approaches were used in this thesis. In one, isotope incorporation and enzyme inductions were performed on the steady state, exponentially growing population, which was then killed, the dead cells fractionated, and the fractions analysed.

The second approach involved fractionation of live cells. Small cells were recovered from the gradient and were subcultured, to provide a synchronously dividing population (Mitchison and Vincent, 1965). Enzyme inductions and isotope incorporations were then performed on aliquots withdrawn from the synchronous culture.

b) Fractionation of Antibiotic treated Cells:

i) Pretreatment and Harvesting of Cells:  

One litre cultures of cells were grown for 15-20 generations until reaching an optical density (540nm) of c. 0.1; about $10^8$ cells per ml.

Prior to harvesting the cells, the desired induction and labelling regimes were performed, and the appropriate antibiotics added. Enzyme inductions were usually of 5-10 minutes duration.

Cells were then rapidly chilled and concentrated by centrifugation, or more rapidly by filtration (Millipore, 0.45 μm pore size, 142 mm diameter filter).

The cells were then suspended in 25-30 mls of cold 3% sucrose, prepared in the growth medium and supplemented with the appropriate antibiotics. This suspension was briefly sonicated (2x1 second at setting 6; MSE sonicator, MSE London) to disrupt any large clumps of cells.
ii) Loading the gradient: Application of the cell suspension:

Fractionation: Collection of Fractions:

A 5-15% sucrose gradient (Prepared in the growth medium and containing the necessary antibiotics) was loaded into the rotor spinning at 400-600 r.p.m. at 4°C. The gradient was delivered from a gradient device by means of a Watson-Marlow peristaltic pump.

The cell suspension was applied, followed by an overlay of 50-100 mls of 1% sucrose.

Acceleration of the rotor to 2,500-3,000 r.p.m. achieved the desired fractionation within 20-40 minutes. Subsequently, the rotor was decelerated, without braking, to 400-600 r.p.m. and the gradient displaced slowly (about 50 mls/minute) into 20 ml fraction tubes on ice.

These steps are detailed explicitly in the following figures.
A. Cross sectional diagram of the zonal rotor. The feed head assembly permits delivery of fluid to the rotor chamber via a high-speed, rotating fluid seal.

Fluid can be delivered to the centre, or edge of the rotor chamber (arrows).

B. Cross sectional detail of the fluid feed channels at the rotor centre.

C. Plan of the base of the rotor chamber. Fluid to the rotor edge passes along channels under the four sector blades. Fluid to the centre emerges along channels in the central perspex turret.

CFL - Centre feed line
CPT - Central perspex turret
EFL - Edge feed line
FHA - Feed head assembly
FS - Fluid seal
LPP - Lower perspex plate
RC - Rotor chamber
RS - Rotor spindle
UPP - Upper perspex plate
• - denotes rubber 'O'-ring seals
These depict operation of the zonal rotor. To illustrate procedures a quadrant of the rotor is shown, together with a graphical representation. The rotor volume is 1300 mls. The sucrose gradient is 5-15%, and linear with respect to rotor volume.

FIGURE 3. (above)

Light sucrose was pumped to the rotor edge from a gradient device. The rotor was spinning at 400-600 r.p.m.
The light sucrose was progressively displaced towards the rotor centre by heavier sucrose from the gradient device, until the rotor was full.

FIGURE 4.
FIGURE 5.

The cell suspension was applied at the top of the gradient by reversing the direction of pumping. Heavy sucrose was withdrawn from the rotor edge, and the cell suspension drawn into the rotor at the centre. The cells were suspended in 3% sucrose.
FIGURE 6.

An overlay of 1% sucrose was applied in the same way as the cell suspension.
FIGURE 7.

After 20-40 minutes at 2,500-3,000 r.p.m., the cells had migrated some way through the gradient.
After deceleration to 400-600 r.p.m., the contents of the rotor were displaced through the centre feed line by pumping 30% sucrose to the rotor edge. 20 ml fractions were collected.
iii) Analysis of the Fractions:

Cells were routinely centrifuged out of the sucrose and re-suspended in a suitable medium, at a suitable cell density. This largely depended on subsequent procedures; where β-galactosidase was to be assayed, the cells were resuspended in 0.9% saline at about $1-10 \times 10^8$ cells/ml. For the assay of tryptophanase, the phosphate-glutathione buffer was used, and the cells were at a density of $10-100 \times 10^8$/ml.

Aliquots of these fractions were suitably diluted into 10% TCA for subsequent scintillation counting; into 0.8% formaldehyde/growth medium for cell counts and photomicrography; and aliquots were also taken for enzyme assays. Optical density was measured directly, or where necessary, on suitably diluted samples.

c) The Sucrose Gradient:  Also See Appendix

Two types of sucrose gradient were used. One was a linear gradient, delivered from a conventional, two chambered, gradient device (Britten and Roberts, 1960). In the rotor, such a gradient, though linear with rotor volume was not linear with respect to rotor radius (Figure 9).

The second gradient used was delivered from a gradient device consisting of a large conical flask containing 1 litre of 5% sucrose. The flask was tightly sealed with a rubber bung, through which two tubes passed. One tube allowed sucrose to be pumped out of the flask, whence heavy sucrose from a reservoir was siphoned into the flask through the second tube. The reservoir contained 1300 mls of 20% sucrose. A magnetic stirrer in the conical flask allowed the sucrose solutions to be mixed.
FIGURE 9.

Loading the zonal rotor with a 5-15% sucrose gradient, delivered from a conventional, two-chambered gradient device.
The sucrose concentration across the rotor is shown, plotted against rotor volume (A) and rotor radius (B).
The sample and overlay are shown.
FIGURE 10.

Loading the zonal rotor with a 5-15% sucrose gradient delivered from the gradient device described in the text.
The sucrose concentration across the rotor is shown, plotted against rotor volume (A) and rotor radius (B).
The sample and overlay are shown.
In the rotor, such a gradient proved to be linear with respect to rotor radius, but not rotor volume (Figure 10).

The latter system was generally chosen, being more convenient in use.

d) Preparation of Synchronous Cultures: Also See Appendix

Exponentially growing cells were fractionated at 20°C in the manner described above. However, a 3-5% linear (with respect to volume) gradient was used, as the fractionation was achieved in about half the time.

20 ml fractions were again collected (the gradient was displaced at about 100 ml/minute) and one judged to contain suitably small cells was inoculated into 150-500 mls of prewarmed growth medium. The resulting culture usually had a starting cell density of about $2 \times 10^7$ cells/ml, and these divided synchronously for at least two generations.

Aliquots were withdrawn at intervals from the culture and pulsed or pulse-chased with radioisotope, or induced for the synthesis of certain enzymes, as previously described.

Subsequent analyses were identical to those described above.

8 One-Dimensional Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis:

a) Preparation and running the gel:

Slab gels were routinely used. Full details of the apparatus have already been described (Studier, 1973).

Briefly, the gel mix was allowed to polymerise in a mould consisting of two glass plates (16 x 18 cm) held 1 mm apart by perspex
strips along three edges. The assembly was clipped together and sealed with vacuum grease and agar.

Before polymerisation of the gel had occurred, a perspex comb was inserted into the top free edge of the mould. Its removal, after polymerisation, left wells in the gel into which samples could be loaded.

For electrophoresis, the comb and bottom perspex spacer were removed. The glass plate/gel sandwich was clipped into an electrophoresis tank such that both the bottom and top edges of the gel were in contact with electrode buffer. The sample wells were filled with electrode buffer.

The samples, in 20% glycerol, were introduced into the wells, below the surface of the buffer, through a fine syringe needle. The glycerol stabilised each sample at the bottom of the sample well.

Electrodes in the upper and lower buffer tanks allowed passage of current through the gel. The samples migrated towards the anode.

Denaturing gels (containing SDS) were used, in which polypeptides were separated on the basis of molecular weight (Laemmli, 1970).

A discontinuous, Davis-ornstein system, was used (Davis, 1964; Ornstein, 1964; Maurer, 1971).

This system incorporated the following components:

The separating gel

The following three separating gels were used

5% acrylamide (BDH)

0.13% bisacrylamide (NN'-methylenebisacrylamide: BDH)

10% acrylamide

16% acrylamide

0.27% bisacrylamide

0.094% bisacrylamide
The running gel buffer was 0.375 N Tris-HCl, pH 8.8.

The acrylamide-bisacrylamide-buffer mixture was degassed under vacuum, and SDS was added to give a final concentration of 0.1%.

The polymerising catalysts were ammonium persulphate and TEMED (N,N,N',N'-tetramethylethylenediamine; Koch-Light Laboratories), both at 0.025% final concentration.

The mixture was poured into the mould described above and gently overlayed with 1 ml of water. Polymerisation took 20-40 minutes.

The running gel (separating gel) was usually about 12-13 cm long.

**The stacking gel**

After the separating gel had polymerised, the water overlay was poured off and the stacking gel (3 cm long) was poured on top. In all cases this gel consisted of:

3% acrylamide
0.08% bisacrylamide
0.0625 M Tris-HCl buffer pH 6.8
1% SDS

This gel was polymerised in the same way as the separating gel.

The perspex comb was inserted into the stacking gel and removed once polymerisation was complete (about 60 minutes).

**Electrode buffer**

0.025 M Tris-glycine pH 8.3
0.1% SDS
0.1% β-mercaptoethanol

**Sample buffer**

0.0625 M Tris-HCl pH 6.8
3% SDS
20% glycerol
5% β-mercaptoethanol
0.001% bromophenol blue, as tracking dye.
Proteins were digested in the sample buffer by incubation at 100°C for 4 minutes

The 5%/0.13% gel was used for analysis of RNA polymerase subunits. In this case pelleted whole cells were digested in the sample buffer prior to electrophoresis.

Analysis of the cell membrane proteins required prior purification of cell membranes (below). The membranes were digested in the sample buffer at 100°C for 4 minutes and electrophoresed on the 10%/0.27% or 16%/0.094% gels.

Electrophoresis was carried out at room temperature, at 6-20 mamps for 10-20 hours.

The functions of the stacking gel and separating gel are amply described elsewhere (Maurer, 1971). Basically, a localised gradient of ionic mobilities in the stacking gel results in the concentration of the sample proteins into a tightly compacted band. In the separating gel the various protein species are separated according to molecular weight.

b) Fixing and Staining the gel:

After electrophoresis, the gels were stained as follows:

i. A period of 10 minutes fixing in 4% methanol/2% acetic acid.

ii. A period of 15 minutes staining in the above solution containing 0.1% Coomassie Blue.
iii. Several hours destaining in successive rinses of 7% acetic acid/5% methanol.

All operations were carried out at 37°C.

Scanning of the stained gels was performed using a Vitatron TLD 100 densitometer (Dieren, Holland).

c) Molecular Weight Determinations:

The method described by Weber and Osborn (Weber and Osborn, 1969) was used.

Standard proteins of known molecular weight were electrophoresed under the same conditions as the protein samples under investigation.

The proteins used as standards were:

- Phosphorylase A of molecular weight 92,000
- Bovine Serum Albumin " " 68,000
- Ovalbumin " " 42,000
- Carbonic Anhydrase " " 29,000
- β-lactoglobulin " " 18,500

d) Slicing, Solubilisation and Radioactive counting of the gel:

The relevant track was carefully cut from the slab gel manually using a sharp razor blade. For transverse sectioning of the track, it was first frozen at -20°C. It was then sectioned into 1 mm slices using either a Mickle gel slicer (The Mickle Laboratory Engineering Company, Comshill, Surrey) or a stack of 150 razor blades, held 1 mm apart by perspex spacers.

The gel slices were solubilised by gentle shaking at 37°C, overnight, in a water-tolerant scintillant, containing a tissue solubiliser.
This scintillant contained -

- 720 mls toluene
- 2.38 gms PPO (2,5, Diphenyloxazole; Sigma)
- 80.8 mgms POPP (1,4-bis-(2-(4-methyl-5-phenyloxazolyl))benzene; Packard).
- 7.2 mls 1% SDS
- 30 mls Soluene\(^{350}\) (Packard).

The radioactivity was counted in a Packard Tri-Carb liquid scintillation counter.

e) **Drying the gels and Autoradiography:**

The distribution of isotope in slab gels was also studied on autoradiograms.

Gels were placed on a sheet of Whatman \#3 filter paper, and dried under vacuum, over a steam bath, (Bonner and Laskey, 1974).

For autoradiography, the dried gel was placed next to a sheet of Kodak Blue Brand medical X-Ray film, BB54, and tightly clamped between two glass plates. This assembly was retained in the dark, at room temperature, for several days.

The autoradiogram was developed using May and Baker Polycon.

9 **Two-Dimensional Polyacrylamide gel electrophoresis:**

Crude cell lysates were analysed by this method, which has already been described in detail (O'Farrell, 1975).

a) **The First Dimension - Isoelectric Focusing:**

Proteins were resolved, in the first dimension, upon rod gels
on the basis of their isoelectric points.

The samples for isoelectric focusing were prepared as follows:—

The cell or membrane pellet was first digested in the SDS-sample buffer described above. To this, an equal volume of lysis buffer was added.

The lysis buffer contained

9.5 M urea
2% Nonidet P-40 (BDH)
2% Ampholines (1.6% of pH range 5-7; 0.4% of pH range 3-10; LKB)
5% β-mercaptoethanol

The urea concentration in the sample buffer/lysis buffer mixture was finally adjusted to 9 M.

The isoelectric focusing gels were prepared as follows:—

The gel mix contained

5.5 gms urea
1.33 mls of acrylamide stock (26.33% acrylamide/1.26% bisacrylamide)
2 mls of a 10% solution of Nonidet P-40
1.97 mls of H₂O
0.4 mls ampholines, pH 5-7
0.1 ml ampholines pH 3-10

The gel mix was degassed and the polymerising catalysts (above) were added.

The mix was loaded into glass tubes sealed at the bottom with parafilm. Each was overlayed with 8 M urea and left for 1-2 hours. The urea was then removed from the top of the polymerised gel and replaced with 20 µl of lysis buffer, which was overlayed with water. The gels were allowed to stand for a further 1-2 hours. The parafilm was then removed, and the glass tubes containing the gels were then placed in
a standard rod gel electrophoresis apparatus. The water and lysis buffer was then removed from the top of each gel. The sample was then applied, followed by 10 μl of sample overlay solution (this contained 9 M urea and 1% ampholines, being 0.8% in the pH range 5-7, and 0.2% in the pH range 3-10). The upper portion of the gel tubes and the upper reservoir of the electrophoresis tank were then both filled with degassed 0.02 M NaCl. The lower reservoir was filled with 0.01 M H₃PO₄.

Electrophoresis was carried out at 400 volts for 17 hours.

b) The Second Dimension:

The rod gels were removed from the glass tubes. Each was then layered on top of a discontinuous, SDS-polyacrylamide slab gel. All components of this system were as described above. A 16%/0.094% gel was used. The rod gel was sealed to the slab gel by a solution of 1% agarose.

Electrophoresis was carried out at 10 mamps for 15 hours. The resulting gel was stained, dried and exposed for autoradiography as described above.

10 Preparation of Cell Membranes:

Bacterial membranes were prepared from sonicated cells by differential centrifugation, largely according to Fox (Fox, Carter and Kennedy, 1967).

All manipulations were carried out at 4°C.

Cells were pelleted by centrifugation at 6,000 g for 5 minutes.

The cells were washed once in a 10 mM Tris-HCl buffer, pH 7.8, containing 5 mM EDTA and 1 mM β-mercaptoethanol, and finally resuspended in the
Cells were lysed by sonication (MSE sonicator, MSE, London; setting 6, 3 x 15 second bursts), and unbroken cells and cell debris were pelleted at 12,000 g for 10 minutes.

The resulting supernatant was centrifuged for 40-60 minutes at 100,000 g in a Beckman L2-65B preparative ultracentrifuge. The resulting membrane pellet was washed in the above buffer, centrifuged again, and finally resuspended in a suitable volume (20-200 µl) of the SDS sample buffer. The membrane proteins were digested in this buffer at 100°C for 4 minutes. Samples were then analysed by SDS-polyacrylamide gel electrophoresis, as described above.
RESULTS

1 Balanced Growth of E. coli B/r:

In order to study cells under defined and reproducible conditions, bacterial cultures were routinely maintained in exponential growth for fifteen or more generations. Under such conditions, cells were in balanced growth. This has been defined by Campbell (Campbell, 1957), who states that "growth is balanced over a time interval if, during the interval, every extensive property of the growing system increases by the same factor".

Figure 11 illustrates this for three "extensive properties" of a culture of E. coli B/r.

Kubitschek (Kubitschek, 1971) has considered balanced growth in terms of a constant cell size distribution in the exponential population. At culture densities greater than $6 \times 10^7$ cells/ml, the cells became smaller, and growth was no longer balanced. It has not been possible to confirm these observations in this laboratory. However, growing cultures were, whenever possible, harvested for zonal fractionation at culture densities of between 5 and $10 \times 10^7$ cells/ml.
FIGURE 11:

Balanced growth of *E. coli* B/r ATCC 12407.

Cells were grown in the minimal salts medium, using glycerol as the carbon source.

A suitably diluted inoculum was chosen such that exponential growth continued for more than fifteen generations.

Cell counts, protein estimations and $O.D_{540}$ were measured on $0.08\%$ sodium azide treated aliquots of the culture.
Separation of E. coli B/r into size classes:

Zonal Centrifugation of Exponentially Growing Cells:

The procedure for separation of exponentially growing cells into size classes has been described in detail in the previous section.

Figure 12 illustrates a typical fractionation, incorporating the procedures discussed below. It can be seen that the mean cell length increased continuously across the rotor, although a two-fold increase was not seen. Fractions containing the larger cells were significantly contaminated with smaller cells.

Certain practical measures were evolved to keep this contamination to a minimum.

It seemed likely that cells would tend to form clumps in the dense suspension applied to the rotor. To reduce this tendency, rarely more than $10^{11}$ total cells were applied. The suspension was also briefly sonicated (2 x1 second at setting 6; MSE sonicator) prior to application to the gradient.

The absence of fimbriae might conceivably further reduce the tendency of cells to form clumps. Figure 13 shows a fractionation of E. coli B/r fim⁻.

The inclusion of 60 units/ml of benzylpenicillin blocked cell division in the asynchronous population (Schwarz, Asmus and Frank, 1969). (The chloramphenicol, which was routinely added, blocked protein synthesis and thus cell elongation.) In so doing, the contamination of larger cells with small cells could be reduced in the zonal fractions. The effect upon mean cell length, for the fim⁻ strain, is indicated in Figure 13a. Penicillin was also included in the experiment shown in Figure 12. Comparison of the two figures indicates that
FIGURE 12: Antibiotic treated cells were separated on the zonal rotor as described in the text. The cell lengths in selected fractions were measured photomicrographically.

a) Mean cell length across the rotor
b) Length distribution of the exponential population
c) Fraction no. 1: mean cell length 1.5 μm: coefficient of variation 11%
d) " " 5 " " " 1.7 " "
e) " " 11 " " " 1.9 " "
f) " " 18 " " " 2.4 " "
g) " " 24 " " " 2.6 " "
FIGURE 13. Zonal separation of E.coli B/r fim. The experimental approach was identical to that for figure 12, but for the exclusion of penicillin as a block to cell division. Mean cell length across the rotor is plotted in figure 13A (Δ). For comparison, figure 13A also shows data from an experiment in which penicillin was included (•). The fraction number (fn), and mean cell length (L), for each distribution are shown.
the afimbriate strain was not resolved any more effectively.

The nature of the sucrose gradient was found not to be critical. The 5-15% gradient, linear with rotor radius, gave marginally improved separations and was convenient in use. This gradient was used in all cases, unless otherwise specified.

The rate at which the gradient was displaced after fractionation was found to be important. Consequently a displacement rate of less than 50 ml/minute was used. This minimised turbulence and viscous drag in the rotor chamber and fluid channels.

These various tactics optimised the separations obtained, although the data from Figure 12 was rarely improved upon.

Photographs of small and large cells from a zonal fractionation are shown in Plates I and II.
PLATES I and II. Separation of bacteria in the zonal rotor.

Plate I is a photograph of small cells taken from near the top of the sucrose gradient. Plate II shows large cells taken from near the bottom of the gradient.
b) Zonal Centrifugation of a mixture of exponential cells and penicillin induced filaments:

The effect of 20 units/ml of benzylpenicillin upon E. coli B/r is shown in Figure 14. Cell lysis was evident after about 60 minutes, as detected by a declining culture turbidity. However, during this time mean cell volume had increased by about 60%.

Figure 15 shows a zonal fractionation of a culture of exponentially growing cells, one half of which had been treated with penicillin at 20 units/ml for about 1 generation. Again, mean cell length increased continuously across the rotor, but over more than a two-fold range. This finding is exploited in a later experiment, in an attempt to define events occurring in the latter third of the cell cycle. Figure 12 indicates that this portion of the cell cycle is not otherwise open to investigation.

Notice the separate uses of penicillin as a division block in these two experimental approaches. In figure 12 the exponential population was treated with chloramphenicol. Penicillin was included to prevent any further cell division, which might otherwise occur, even in the absence of protein synthesis. This procedure tended to reduce the contamination of large cell containing fractions with newly divided cells.

In Figure 15 the exponential cells were grown in the presence of penicillin. Protein synthesis was inhibited after about one generation (by which time cell elongation had occurred), and the cells were harvested and fractionated. This procedure allowed a greater than two-fold cell size range to be studied.
FIGURE 14.

The effect of low penicillin concentrations upon E. coli B/r.

The arrows indicate the time of addition of 20 units per ml of benzylpenicillin to the growing culture.
FIGURE 15. Zonal separation of a mixture of exponentially growing cells and penicillin induced filaments.
c) **Assessment of the efficiency of Zonal Fractionation:**

Scoring cell size distributions photomicrographically did not provide a rapid means of assessing a given zonal separation.

A convenient index of mean cell size is the O.D./cell number ratio for a sample of cells in liquid medium. This ratio was found to increase continuously across the rotor in the same way as the more laboriously determined mean cell length. (Figure 16). Repeated experiments have not allowed a distinction to be drawn between a linear or logarithmic increase in cell mass during the cell cycle. (Kubitschek, 1969; 1970; this thesis, Figure 18).

An alternative index of zonal separation was the percent dividing cells in each fraction. Figure 16 also shows this parameter across the rotor.

d) **Distribution of cell numbers across the rotor:**

It has been shown, that for *Saccharomyces cerevisiae*, the distribution of cell numbers per fraction across the rotor, corresponds closely to the cell size distribution of the original exponential population (Sebastian, Carter and Halvorson, 1971).

This correspondence is shown for *E. coli* B/r in Figure 17.
Size separation in the zonal rotor. Mean cell size, and the percent dividing cells, were estimated for selected fractions from a typical zonal separation.
FIGURE 17.

Figure 17A shows the cell length distribution of the exponential population growing in the minimal-glycerol medium.

Figure 17B shows the cells per ml for successive fractions across the zonal rotor.

Photomicrography was performed on cells fixed in the formaldehyde/growth medium described.

Azide treated cells were fractionated on the zonal rotor, and cell counts performed directly on the fractions obtained.
Determination of Cell Age in Zonal Fractions:

a) Protein and Enzyme Content:

The exponentially growing cell, in balanced growth, would be expected to double its every "extensive property" within one generation time. If size classes also represent age classes, this expectation may be demonstrated in the zonal size separations.

Figure 16 has shown that cell volume increased across the zonal rotor. Figure 18 shows that cellular protein content increased similarly.

The cells (fig. 18) were fully induced for β-galactosidase synthesis, having been grown for more than fifteen generations in the presence of $10^{-3} \text{ M IPTG}$. Figure 18 shows cellular enzyme content across the rotor. (See also Figure 1a.) The cells were also pulse labelled with tritiated leucine for 4 minutes. Incorporation of isotope into TCA precipitable material, as a measure of the rate of protein synthesis, is also shown in Figure 18.

Clearly, every extensive property has increased continuously across the rotor to the same extent as the mean cell volume.
FIGURE 18. Macromolecular syntheses during the cell cycle. Cells grown in the presence of $10^{-3}$ M IPTG were pulse labelled for 4 minutes with 1 μCi/ml of $^{3}$H-leucine (57 Ci/mmol) plus unlabelled leucine at 1 μg/ml. The pulse was chased for 3 minutes with 200 μg/ml of unlabelled leucine, and uptake finally terminated with 200 μg/ml of chloramphenicol. Cells were then separated in the zonal rotor.

Enzyme activity, isotope incorporation and mean cell size across the rotor are shown.

Total protein was estimated by the Lowry method in a separate experiment.
b) **Cell Cycle Markers:**

It was not possible to chart the temporal order of cell cycle events on the basis of any of the above data. Nor was it possible to locate precisely the timing of cell division. The use of internal cell cycle markers (Mitchison, 1969b) was therefore necessary.

The changes in rates of DNA, β-galactosidase, D-serine deaminase and tryptophanase synthesis, as a function of time in the cell cycle, have been defined in *E. coli* B/r (Helmstetter, 1968; Pato and Glaser, 1968). The data of Helmstetter is schematised in Figure 19. These known changes in rates of specific macromolecular syntheses, provide useful markers of cell age in cell cycle studies. (It is likely, however, that the rate change for D-serine deaminase synthesis is wrongly placed; see Introduction.)

These changes in the rates of synthesis of DNA, β-galactosidase and tryptophanase were studied in zonal fractionations of *E. coli* B/r. The results are shown in Figures 20 and 21. Figure 22 shows similar data from an experiment of the type shown in Figure 15.

It is not easy to relate these markers to the cell division cycle. However, if cells of age 0.0 are assumed to have a cell volume of 70 O.D./cell units, then cell age across the rotor can be deduced. It is further assumed that the Half-Doubling Time (or Half-Rise Time, where a complete rate doubling is not observed) of a given rate change, represents the discrete time in the cell cycle at which the rate change under study, actually occurred.

Accordingly, the change in the rate of DNA synthesis was found to occur at a cell volume of 115 units, or cell age 0.65 (Figure 20). The change in rate of β-galactosidase synthesis occurred at cell volumes of 95 (Figure 20) and 90 (Figure 21), or at cell ages of
FIGURE 19. The temporal order of events in the bacterial cell cycle.

The changes in the rates of synthesis of β-galactosidase (Lac), D-serine deaminase (Dsd) and Tryptophanase (Tna) are shown, relative to both the cell division cycle and the DNA replicative cycle. (I and T denote initiation and termination of DNA replication.)

Based on the data of Helmhstetter (Helmstetter, 1968).
FIGURE 20.

The rates of synthesis of β-galactosidase and DNA during the bacterial cell cycle.

Enzyme synthesis in the exponential culture was induced for 6 minutes with $10^{-3}$ M IPTG, and the induction terminated with 200 μg/mL of chloramphenicol. During the last 90 seconds of the induction, $^3$H-thymidine (25 Ci/mmol; 5 μCi/mL) was added to the culture. Incorporation was terminated with 20 μg/mL of nalidixic acid. Penicillin was added at 60 units per mL.

The cells were separated in the zonal rotor. Enzyme activity and isotope incorporation in each fraction were measured.

The dotted line represents the mean cell size across the rotor, in terms of O.D./10$^{11}$ cells. The arrows indicate the cell size at which the changes in rates of macromolecular synthesis occur, (page 48).
FIGURE 21.

The rates of synthesis of β-galactosidase and tryptophanase during the bacterial cell cycle.

The synthesis of both enzymes was induced simultaneously for 5 minutes, by addition of $10^{-3}$ M IPTG and 1 mgm/ml of L-tryptophan, to the exponential culture. The inductions were terminated by addition of 200 μgm/ml of chloramphenicol. Penicillin was added at 60 units/ml.

The cells were separated in the zonal rotor and the enzyme activity in the fractions was assayed.

The dotted line represents the mean cell size across the rotor. The arrows indicate the cell size at which the changes in rate of enzyme synthesis occur.
FIGURE 22.

The rate of synthesis of DNA and tryptophanase across the rotor: inclusion of penicillin induced filaments in the fractionation.

One half of an exponentially growing culture was treated with 20 units/ml of benzylpenicillin for about half a generation time. The penicillin treated cells were then added to the untreated cells, and the induction and labelling regimes used previously were carried out. Chloramphenicol and nalidixic acid were added, and the cells were separated in the zonal rotor.

The rates of DNA, and tryptophanase synthesis, and the mean cell size across the rotor are shown.
0.35 and 0.29 respectively. The change in rate of tryptophanase synthesis occurred at a cell volume of 110 units, or cell age 0.6 (Figure 21).

Cell age cannot be estimated in Figure 22 for the markers shown. However, the increase in the rates of synthesis of DNA and tryptophanase occurred simultaneously.

These data, together with corresponding data from synchronous cultures, are discussed later, and compared with the data of Helmsatter.

c) **Synchronous Cultures:**

Selection of a discrete size class of cells from a zonal fractionation of live cells, and subculturing in fresh medium, should produce a synchronous culture (Mitzhison and Vincent, 1965; Halvorson, Carter and Tauro, 1971b).

This approach has been used as an indicator of cell age in a given zonal fraction. Sebastian et al (Sebastian, Carter and Halvorson, 1971), have shown that for Saccharomyces cerevisiae, selection of small cells from a zonal fractionation, yielded a synchronous culture which divided after one generation time had elapsed. These were therefore young cells. The largest cells, which were also the oldest, likewise produced a synchronous culture, but division occurred immediately.

Beck and Park (Beck and Park, 1976) selected small cells from a zonal fractionation of E. coli D11 (a K12 strain). These were inoculated into fresh medium, and a synchronous culture resulted. However, cell division occurred after only 0.65 of a generation. Beck and Park concluded that these cells were of age 0.35 to begin with.
Figures 23, 24 and 25 show synchronous cultures of *E. coli* B/r. The inoculum was taken from near the top of the sucrose gradient, and contained the smallest cells. After a brief lag upon reinoculation into fresh medium, cell division ensued after 1.0 generation time. The inoculum therefore contained cells of age 0.0, as would be expected.

4 The Cell Cycle in Synchronous Cultures:

a) The DNA-Division Cycle:

The method of zonal separation of exponentially growing cells does not permit the relationship between cell cycle markers and cell division to be observed. Conventional synchronous cultures, or the membrane elution technique of Helmatetter (Helmatetter and Cooper, 1968) allow this relationship to be examined.

Helmatetter and Cooper (Helmatetter and Cooper, 1968; Cooper and Helmatetter, 1968; Helmatetter, 1967) have made a thorough study of this relationship at various growth rates in the B/r organism also studied in this laboratory. They have shown that initiation of DNA replication begins a cell cycle in which subsequent cell division occurs after approximately 60 minutes. Replication of the chromosome takes forty minutes. Cell division occurs twenty minutes after termination of DNA replication. For cells grown in the minimal salts medium, containing glycerol as sole carbon source, initiation of DNA replication occurs at about cell age 0.8–0.9.

Synchronous cultures of *E. coli* B/r were produced as described under Materials and Methods. The rate of DNA synthesis in these cultures was expressed in terms of the incorporation of tritiated thymidine, following a 1 minute pulse, into TCA precipitable material. The data from such experiments are shown in Figures 23, 24 and 25.
The relationship between cell division, and initiation and termination of DNA replication.

A synchronous culture was produced as described under Materials and Methods.

Figure 23A shows the rate of DNA synthesis in such a culture. Aliquots (1ml) were withdrawn from the culture onto 5 μCi (25 Ci/mmol) of $^3$H-thymidine. After 1 minute, the isotope incorporation was stopped by addition of 1 ml of 10% TCA containing 200 μgm/ml of unlabelled thymidine. The TCA ppt. was collected on a millipore filter and the radioactivity counted. The arrow indicates the HDT for the doubling in rate of DNA synthesis; this represents initiation (I) of DNA replication.

Figure 23B shows the cells/ml in the synchronous culture, and in chloramphenicol treated samples, (page 51). The time of cell division is indicated as DIV. The estimated time of termination of DNA replication (T) is also shown.

Plus chloramphenicol ■
Minus chloramphenicol ●
FIGURE 2.

β-galactosidase and DNA synthesis during synchronous growth of E. coli B/r.

The synchronous culture was produced as described previously. Cell division (DIV) is estimated from the cell number curve (●).

The rate of DNA synthesis (■) was estimated as described in the previous figure. The HDT for the rate change is indicated, and the time of its occurrence expressed relative to the cell division cycle.

The potential for β-galactosidase (▼) synthesis was estimated by assaying enzyme activity in aliquots induced with 10^{-3} M IPTG for 8 minutes. The HDT for the potential doubling is shifted 6 minutes to the right (page 52), and this is indicated together with the cell age, above (PGz).
FIGURE 25.

Enzyme synthesis during synchronous growth of *E. coli* B/r at 41°C.

The synchronous culture was produced as previously described. The DNA-Division cycle is indicated on the graph.

Enzyme was induced for 9 minutes in aliquots withdrawn from the culture. The potential for tryptophanase (Tna) and D-serine deaminase (Dsd) synthesis is shown.
It was not possible to determine directly the time at which termination of rounds of replication took place. In order to make this determination, aliquots of the synchronous culture were removed at timed intervals, onto 200 µg/ml final concentration of chloramphenicol, and incubation at 37°C continued for 90 minutes.

Cells which have completed DNA replication will divide in the presence of chloramphenicol; those still undergoing DNA replication at the time of addition of inhibitor will not divide (Jones and Donachie, 1973; Pierucci and Helmstetter, 1969).

At the end of the 90 minute incubation, above, cell numbers were determined. The number of cells per ml in the chloramphenicol treated samples were plotted, at the time of their addition to chloramphenicol, in Figure 23. Thus the time of termination of DNA replication can be determined.

It was not possible to distinguish between initiation and termination of DNA replication in these cultures. It can only be concluded that the initiation/termination event occurs about 20 minutes before cell division, and that this event occurs with the same frequency as cell division, i.e. every 56 minutes (fig. 24): every 50 minutes (fig. 25).

b) **Cell Cycle Markers:**

Enzyme potential (as an index of the rate of enzyme synthesis), and its relation to gene dosage has been discussed in the Introduction. It was noted that this relationship had been established without adequate knowledge of the origin and direction of DNA replication.

Potential for the synthesis of β-galactosidase, tryptophanase and D-serine deaminase during synchronous growth of *E. coli* B/r is
shown in Figures 24 and 25.

The Half-Doubling Time (HDT) for the change in rate of DNA synthesis is indicated, together with that for cell division.

The enzyme activity, accumulated during an 8-12 minute induction, is plotted at the time of addition of inducer to the cells. It is preferable to plot the enzyme activity at the mid-point of its accumulation during the induction period, (Schmidt, 1974a; 1974b). Hence the HDT's for the changes in rate of enzyme syntheses are displaced 5-6 minutes to the right.

The cell ages at which the various cell cycle events occur are shown in Figure 26, together with the corresponding data from the zonal rotor.

These findings are considered more fully in the Discussion section.
DIAGRAMMATIC SUMMARY OF THE TIMING OF CELL CYCLE EVENTS INVESTIGATED IN THE PREVIOUS EXPERIMENTS.

The changes in rates of synthesis of DNA, β-galactosidase (βGz), tryptophanase (Tna) and D-serine deaminase (Dsd), during the cell cycle are shown. The data is related both to the cell division cycle, and the period of DNA replication derived from the experiments with synchronous cultures. No period could be discerned in which DNA replication was not taking place.

The long, double-headed arrows indicate average values derived from the various separate determinations made (short arrows). The data derives from both zonal fractionations and synchronous cultures.
c) The synthesis of RNA Polymerase and β-Galactosidase during synchronous growth:

Plate III shows a 5%/0.13% SDS polyacrylamide gel separation of cell proteins, derived from both induced and uninduced cells. β-galactosidase had been induced with 10^{-3} M IPTG, for several cell generations. The gel bands corresponding to β-galactosidase and the β and β' subunits of RNA polymerase are indicated. This separation technique provides a means to estimate enzyme protein in radioactively labelled preparations, (Matzura, Hansen and Zeuthen, 1973).

Figure 27 shows a control experiment in which the recovery of labelled ββ' from the gel was related to the amount of lysate applied to the gel. ββ' radioactivity represents about 1.1% of the total loaded onto the gel. Matzura et al have called this percentage the ωₚ ratio. For pulse-chase labelled preparations it expresses the rate of enzyme synthesis as a fraction of the rate of total cell protein synthesis. Matzura et al derived an ωₚ value of about 1.2% in glucose grown cells of E. coli K12.

If the total rate of protein synthesis is known, then the specific rate of enzyme synthesis can be derived from a knowledge of the ωₚ ratio. In synchronous systems, the rate of synthesis per ml is more easily interpreted than the ωₚ ratio (Mitchison, 1971; this thesis, Discussion).

Since β-galactosidase is readily detected on these 5%/0.13% gels, its rate of synthesis can be determined easily in pulse-chase labelled preparations. The rate of synthesis can also be measured in terms of the potential for synthesis. These two methods for determining the rate of β-galactosidase synthesis are compared in Figure 28, for exponentially growing cells.
PLATE III.

Separation of proteins on a 5%/0.1% SDS-polyacrylamide gel. Cells were either induced or uninduced for the synthesis of β-galactosidase. The cells were pelleted by centrifugation, digested in the SDS sample buffer, and the lysates examined by gel electrophoresis.

The uninduced samples are indicated (arrows). The β and β' subunits of RNA polymerase, and β-galactosidase are indicated.
Exponentially growing cells were pulse labelled for 2 minutes with $^3$H-leucine (43 Ci/mmol; 1 µCi/ml) in the presence of 0.1 µg/ml of exogenous unlabelled leucine. The isotope was chased into completed polypeptide chains with 200 µg/ml of exogenous unlabelled leucine. Incorporation of leucine was terminated with 200 µg/ml of chloramphenicol. The cells were pelleted, washed and digested in SDS-sample buffer. Aliquots of this crude lysate (2-20 µls) were run on a 5%/0.1% SDS-polyacrylamide gel. The $\beta$ and $\beta'$ subunits of RNA polymerase were recovered from the gel. The radioactivity in the two subunits was measured and compared to the total label in the equivalent volume of the crude lysate. $\beta$ and $\beta'$ represented 1.1% of the total label in the lysate.

**FIGURE 27.**

Recovery of purified protein from an SDS-polyacrylamide gel.
Measuring the rate of $\beta$-galactosidase synthesis. Two exponential cultures were grown. One contained IPTG at $10^{-3}$ M (●), the other did not (▼). From the first, aliquots were pulse-chase labelled with leucine as described in the previous figure. The rate of $\beta$-galactosidase synthesis, relative to total protein synthesis was determined, in the same manner as the relative rate of $\beta\beta'$ synthesis in the previous figure. This value, times the O.D. 540 of the culture, is plotted above (o).

In the uninduced culture, enzyme potential was measured in the same way as in the synchronous cultures shown previously. The data is presented above (▼).

The cross bars represent the duration of the $^3$H-leucine and IPTG pulses, as indicated.
In the analysis of RNA polymerase synthesis, it was found that the rates of β and β' synthesis were not equal. This was an artefact, caused by the co-migration of an unknown, contaminating protein species, with the β subunit during electrophoresis (R. Hayward, pers. comm.). Accordingly, in the study of RNA polymerase synthesis during the cell cycle (Figure 29), only the rate of β' synthesis is considered. Figure 29 also shows the rate of β-galactosidase synthesis, as well as the rate of total protein synthesis, during the cell cycle. The times of cell division, and the HDT for the change in rate of DNA synthesis are indicated.

The change in rate of DNA synthesis occurred later in the cycle than previously established in this thesis - in fact at about cell age 0.8. The change in the rate of β-galactosidase synthesis also occurred later, at about cell age 0.45. Normalising the data for DNA synthesis brings the β-galactosidase HDT to cell age 0.35. These data are shown in Figure 26.

There is no discernible effect of gene dosage upon the synthesis of the β' subunit. Any such effect would be expected to be manifest at around the time of Initiation of DNA synthesis, since the β' structural gene is at 79' on the E. coli chromosome (Taylor and Trotter, 1972).

The synthesis of both enzymes was seriously perturbed by the synchronisation procedure. To a lesser extent, total protein synthesis was also perturbed.
Synthesis of RNA polymerase and β-galactosidase during the bacterial cell cycle.

A synchronous culture of E.coli B/r was established. Aliquots were removed at intervals and pulse-chased with $^3$H-leucine as described in figure 27. The incorporation of isotope into TCA precipitable material (o) is a measure of the rate of protein synthesis in the synchronous culture. The pulse-chased aliquots were analysed by SDS-polyacrylamide gel electrophoresis, and the relative rates of synthesis of β-galactosidase and the β' subunit of RNA polymerase, thus determined. The percentage values obtained, times the rate of protein synthesis, represent the rates of enzyme synthesis per ml of the synchronous culture.

Prior, during and after the zonal fractionation, the cells were exposed to 10⁻³ M IPTG.
The Synthesis of Membrane Proteins during the Cell Cycle:
Preparation, and SDS-polyacrylamide gel electrophoresis of membrane proteins has been described under Materials and Methods.

Figure 30 shows a densitometer tracing of a typical electrophoresis profile of membrane proteins upon a 10%/0.27% polyacrylamide gel. Marker proteins of known molecular weight were electrophoresed in the same experiment. The molecular weight values are indicated.

Jones and Donachie (Jones and Donachie, 1973) have shown that a brief transcriptional event, upon termination of DNA replication, is a prerequisite of bacterial cell division. Jones (Jones, 1974) has identified two membrane proteins of molecular weight 60,000 and 20,000 (TAP 1 and TAP 2) which were synthesised (or inserted into the membrane) only at the time of termination of replication.

Churchward and Holland (Churchward and Holland, 1976) claim that a 76,000 molecular weight membrane protein is synthesised only at the time of cell division.

Dix and Helmstetter (Dix and Helmstetter, 1973) have shown that protein synthesis, at or near the time of termination of DNA replication, is necessary for cell division.

It seemed relevant to current models of the association between protein synthesis, termination of replication, and cell division, to look for termination associated proteins during the cell cycle of the B/r strain studied by Dix and Helmstetter.

Zonal fractionation was the method of choice. Most importantly the method allows the study of unperturbed cells, which was not so in the system used by Jones.

A control double-labelling experiment was first performed to
FIGURE 30.

Purified membrane proteins from exponentially growing *E. coli* B/r.

The membrane proteins were resolved by SDS-polyacrylamide gel electrophoresis (10%/0.27% gel). The gel was stained and scanned as described under Materials and Methods.

Protein standards of known molecular weight were co-electrophoresed, and their molecular weights are indicated.

The bromophenol blue (EPB) dye front is indicated.
assess the effect of osmotic shock on the membrane proteins. Cells labelled with tritiated leucine were suspended in 10% sucrose, at 4°C, for 40 minutes. They were then washed and resuspended in 0.9% saline. These cells were then mixed with untreated cells labelled with 14C leucine. Membranes were prepared, and SDS-polyacrylamide gel electrophoresis performed as described. The gels were sliced and solubilised as described and the radioactivity measured in a liquid scintillation counter. The 14-Carbon and tritium profiles were identical.

In an attempt to discover possible termination associated proteins in the membranes of E. coli B/r, exponentially growing cells were pulsed for 4 minutes with 3H leucine, and chased for 3 minutes with excess cold leucine. Leucine uptake was then terminated by the addition of chloramphenicol. The cells were chilled, concentrated and separated into size classes by zonal centrifugation. Figure 18 shows the characteristics of this separation.

Fractions, as indicated on Figure 31, were pooled, and each mixed with exponential cells labelled with 14C leucine. Membranes were prepared, electrophoresis (10%/0.27% gel) performed, gels stained, sliced and solubilised, and the radioactivity was measured in each gel slice, all as previously described; Materials and Methods.

Figures 32, 33, and 34 show the double label profiles for each of the three gels. All profiles were essentially identical. At no time was the exponential population found to contain any 14C labelled species which was not also present in the 3H labelled, fractionated cells. This finding is particularly relevant in Figure 32, where the 3H labelled fraction contained 0% dividing cells, compared with 15-20% in the 14C labelled exponential population.
FIGURE 31.

Fractions used in the analysis of the synthesis of membrane proteins during the bacterial cell cycle.

The zonal separation was that described in figure 18. The above graph shows the percent dividing cells across the rotor.

The cross bars indicate those fractions which were selected for the analysis of membrane proteins. Odd-numbered fractions only were taken and pooled. Each group is designated small, medium, or large, according to the cell size in the pooled fractions.
FIGURE 32.

Synthesis of membrane proteins during the cell cycle.

Exponentially growing cells were labelled with $^{14}$C-leucine (34.2 mCi/mmol: 5 μCi/ml) for 4 minutes, and chased with unlabelled leucine (200 μgm/ml) for 3 minutes. Chloramphenicol was then added at 200 μgm/ml. These cells were mixed with the "small" cells from figure 31.

Cell membranes were prepared, and analysed by SDS-PAGE on a 10%/0.27% gel.

The gel was cut into successive 1 mm slices and the double label in each slice counted, and plotted above as a percentage of the total $^{14}$C or $^3$H counts in the gel.

Molecular weights, estimated from the co-electrophoresis of known marker proteins, are indicated.
FIGURE 33.

The procedure was identical to that in figure 32, except that "medium" cells from figure 31 were used.
FIGURE 34.

The procedure was identical to that in figure 32, except that "large" cells from figure 31 were used.
Termination Proteins were therefore not detected in these experiments.

It must be noted that the tritium labelled cells were fully induced with IPTG. Consequently, the $^3$H labelled lac permease protein should be detectable in the membrane profiles.

Jones and Kennedy (Jones and Kennedy, 1968) performed a similar experiment on E. coli ML306, in which separately labelled membrane proteins from induced and uninduced cells were analysed by SDS-polyacrylamide gel electrophoresis. They found that the induced cells contained a membrane protein of molecular weight 30,000, which constituted $3\%$ of total membrane protein. This was shown to be the lac permease.

My own profiles have not detected the permease at the 30,000 molecular weight position. However, all profiles show two excess peaks of tritium counts at 36,000 and 80,000 molecular weight. The excess of tritium at 36,000 represents about $3\%$ of the total in each gel profile. This could possibly be the lac permease. It is not known what the excess tritium at the 80,000 position is due to.

This methodology is unfortunately sensitive only to large $^{14}$C, $^3$H differences. Resolution of membrane proteins is greatly increased upon the $16\%/0.094\%$ gels (Lutkenhaus, pers. comm.). Using this gel system we have further investigated the synthesis of membrane proteins during the cell cycle of E. coli B/r. Again cells were resolved into size classes by zonal centrifugation, following a 4 minute pulse of $^{35}$S methionine and a 3 minute cold methionine chase. Membranes were prepared from selected fractions, and gel electrophoresis performed. The gel was then dried down under vacuum and autoradiography performed as described under Materials and Methods. Plate IV shows a photograph
PLATE IV. Synthesis of membrane proteins during the cell cycle.

An exponential culture of *E. coli* B/r was pulse-chased with $^{35}$S-methionine, as described in the text. The cells were fractionated in the zonal rotor, and membranes prepared from selected fractions. These were analysed by gel electrophoresis (16%/0.094%) and autoradiography, (above). The percent dividing cells in the fractions studied, is indicated at the top of each gel track; E denotes the exponential population. The standard protein markers are indicated: p (phosphorylase A, m.wt. 92,000), b (bovine serum albumin, m.wt. 68,000), o (ovalbumin, m.wt. 42,000), c (carbonic anhydrase, m.wt. 29,000), La (β-lactoglobulin, m.wt. 18,500), Ly (lysozyme, m.wt. 14,000).
of one such autoradiograph. Again, at no time during the cell cycle was any membrane protein exclusively synthesised. This finding has been confirmed in autoradiographs of $^3$H leucine labelled membrane proteins (Lutkenhaus, pers. comm.).

The increased resolution of the 16% gel; the ability to compare large with small cells, rather than exponential with small cells; and the clarity with which minor proteins can be detected, all render this system much more sensitive than the sliced gel analysis used above. Even so, none of the previously reported, periodically synthesised termination (Jones, 1974), or division (Churchward and Holland, 1976) associated proteins, could be detected using this method.

6 Two-Dimensional Polyacrylamide Gel Analysis of Proteins Synthesised during the Cell Cycle:

Total cell protein was analysed by two-dimensional gel electrophoresis and autoradiography. Lutkenhaus and Richardson (unpublished data) have used this technique to study the rates of synthesis of cell proteins during the cell cycle. Samples were pulse-chased with $^{35}$S methionine, as previously described. Crude cell lysates were subjected to two-dimensional gel electrophoresis, and the incorporation of the isotope into the many proteins thus resolved, was recorded autoradiographically.

Many such autoradiographs have been prepared during various cell cycle studies. Lutkenhaus and Richardson have examined both membrane and whole cell proteins in this way, on samples taken from both synchronous cultures and zonal fractionations. At no time during the cell cycle have they been able to detect any uniquely synthesised protein species.
Plates V, VI and VII are photographs of three autoradiographs of such 2-D gels. The gels are of crude cell lysates from small, large and dividing cells taken from a synchronous culture of *E. coli* B/r.

Recording the distribution of isotope upon these 2-D gels in this way does not permit a quantitative analysis of the relative rates of synthesis of the various protein species during the cell cycle. However, if any proteins were synthesised at only one time during the cell cycle, they should be detectable qualitatively using this technique. Scrutiny of the various autoradiographs has yet to reveal any such periodically synthesised proteins.

Cooper (pers. comm.) has studied protein synthesis during the B/r cell cycle in this way, using the membrane elution technique. He too has not observed any periodically synthesised proteins.
PLATES V, VI and VII. Protein synthesis during the bacterial cell cycle.

A synchronous culture of *E. coli* B/r was established. Aliquots were withdrawn at intervals and pulse-chased with $^{35}$S-methionine. Crude cell lysates were prepared and analysed by 2-D gel electrophoresis. The radioactivity in each gel was recorded autoradiographically. The above plates are photographs of the autoradiographs.

Plate V derives from young, newly divided cells.
Plate VI derives from older cells, prior to cell division.
Plate VII derives from cells undergoing division.
DISCUSSION

1 Fractionation of cells into size classes:

A substantial portion of this thesis has been devoted to the development of the technique of size fractionation of *E. coli* B/r, using the zonal rotor.

The method has not been fully exploited in the study of prokaryotic organisms, consequently there is little published data with which to draw comparisons. Sebastian et al (Sebastian, Carter and Halvorson, 1971) have carried out a thorough study of the method and its applications, in their experiments on the *Saccharomyces cerevisiae* cell cycle.

There are several criteria against which the efficiency of size resolution may be judged.

Sebastian et al have argued that the size distribution of the members of an exponential population should closely compare to the distribution of cell numbers in the successive fractions across the rotor, if the separation is principally on a size basis. They have shown this correspondence to be extremely close for *S. cerevisiae*.

Tan et al (Tan et al, 1974) have examined this same aspect of their zonal fractionations of *Myxobacter* AL-1. The correspondence between the two distributions was less precise than for *S. cerevisiae*, with a "tailing" of cells on the right hand side of the number distribution. This "tail", at the bottom of the gradient, characteristically contained cells of all sizes, which had not been resolved by the fractionation procedure.
Beck and Park (Beck and Park, 1976) have not examined this criterion, but they do report that fractions from the bottom of the gradient contained heterogeneous cell populations.

The relationship between the distribution of cell numbers across the rotor, and the size distribution of the exponential population, for *E. coli* B/r is shown in figure 17. The data is similar to that of Tan et al, the cells in the "tail" being apparently unresolved in the fractionation.

A second criterion of size fractionation is that of the size distribution of cells in representative fractions across the rotor. Beck and Park have surprisingly published no data for this aspect of their work. However, fractions from the bottom of the gradient were found to contain heterogeneous cell populations. These fractions contained cells which had more than one constriction and were longer than two cell lengths. Furthermore, synchronous cultures established from these fractions failed to show a complete doubling of cell number in the first division. Therefore these fractions must have also contained a significant number of small or undividing cells.

Tan et al have published cell length distributions of four fractions from a zonal separation. However, no statistical analysis was performed.

Sebastian et al have given explicit details of the volume distributions of cells from representative zonal fractions of *S. cerevisiae*. The coefficient of variation about the mean volume for each fraction varied from 15-20%. These cell volume distributions were measured using an early Coulter size plotter. Thus it is likely that the true coefficients of variation for these distributions were
less than those published (Harvey, 1968).

The length distributions of *E. coli* B/r, in zonal fractions, are shown in figure 12. Statistically the distributions are similar to those reported for *S. cerevisiae*.

A third criterion of successful size fractionation is the mean cell length, or mean cell size, across the rotor. I have rarely obtained more than a 60-70% increase in either parameter across the rotor.

The increase in mean cell length discernible from the data of Tan et al. is about 50%.

Sebastian et al. report a 100% increase in mean cell volume across the rotor in their fractionations of *S. cerevisiae*. Beck and Park report a similar finding in their fractionations of *E. coli D-11*.

I have found that the inclusion of penicillin induced filaments in these zonal fractionations allows resolution of cells over more than a two-fold size range, (figure 15). It seems unlikely, however, that this approach is of any real help in analysing cell cycle events which occur near the end of the division cycle.

I regularly found that the larger cells from the bottom of the density gradient were markedly contaminated with smaller cells. This may have been for several reasons.

Firstly, clumps of cells of all sizes might migrate rapidly through the gradient and dissociate into their constituent particles. Secondly, cells of all sizes might be physically pushed to the bottom of the gradient by the migrating cell boundary, during fractionation (Sebastian, Carter and Halvorson, 1971). To reduce these possibilities
the cell suspension was briefly sonicated before application to the gradient, and fewer than $10^{11}$ cells were applied. Interestingly, Beck and Park routinely loaded their gradients with about twenty times this number of cells.

Alternatively, the presence of small cells at the bottom of the gradient may result from division of those cells already close to division at the time of harvesting. The inclusion of low concentrations of penicillin, as a block to cell division, was thought to aid resolution (figure 13a), although the benefit of this procedure proved to be rather variable.

Some of the heterogeneity in the fractions must have arisen from turbulence in the gradient, due to rotor wobble and perhaps viscous drag upon displacement of the gradient. In an attempt to reduce this latter effect, fractions were collected by slow displacement of the gradient; about 50 mls/minute.

However, there are certain intrinsic imperfections of the fractionation procedure which cannot be avoided. The first concerns the density of the particles to be fractionated.

The sedimentation velocity, $V$, of a perfect sphere of radius $a$, and density $p$, through a medium of density $p_1$ and viscosity $\eta$, subject to a centrifugal force dependent upon the distance, $r$, of the sphere from the axis of rotation, and the angular velocity, $\omega$, is given by the equation

$$V = \frac{a^2(p - p_1) \omega^2 r}{18 \eta}$$

Clearly, the separation of particles is dependent upon their density as well as their size. It is not known whether the density of \textit{E. coli} alters during its cell cycle.
Secondly, it is difficult to predict how a bacterial rod would behave in a density gradient. Conceivably, a long rod would tend to align its long axis in the plane of the g force acting upon it. Consequently the viscous drag of the medium upon rods of different lengths may well be quite similar.

Budding yeasts and mammalian cells which approximate the spherical form, are perhaps therefore better suited to this method of size resolution.

2 Fractionation of cells into age classes: Also See Appendix

The efficiency of resolution of cells into size classes has been discussed. However, it is necessary to show that these size classes correspond to age classes.

Rigorous mathematical analyses of the statistics of the cell division process, and the relationship between cell size and cell age, have been provided by several authors (Harvey, Marr and Painter, 1967; Koch and Schaechter, 1962; Koch, 1966; Kubitschek, 1968).

It is nevertheless necessary, in each zonal separation, to be able to estimate cell age across the rotor. This is best done by examination of convenient, known, cell cycle markers (Mitchison, 1969b).

Tan et al have produced no data which would clearly indicate cell age in their zonal fractions.

Beck and Park established synchronous cultures from cells at the top (small cells) and bottom (large cells) of the sucrose gradient. Their smallest cells divided after a lag of 45 minutes, their largest cells after a lag of 10 minutes. The generation time of their strain was 70 minutes. Thus the smallest cells from the gradient were of age 0.35, whilst the largest were of age 0.95. However, it was also shown
that the mean cell volume of the largest cells was twice that of the smallest. On this basis it would seem that cell size and cell age were not closely correlated. No known cell cycle markers were studied by Beck and Park.

Sebastian et al (Sebastian, Carter and Halvorson, 1971) have studied the synthesis of DNA, α-glucosidase and Ornithine transaminase during the cell cycle of *Saccharomyces cerevisiae*, in both synchronous cultures and zonal fractionations.

The zonal separations confirmed that DNA synthesis occurred during the middle third of the cell cycle (Williamson and Scopes, 1960). The characteristic stepwise pattern of enzyme activity during the yeast cell cycle were found for the above enzymes, both in the zonal separations and synchronous cultures. The enzyme steps occurred in the middle of the cell cycle in both cases. Thus it was shown that the cells were distributed across the rotor according to age as well as size.

Similar attempts were made to correlate cell size and cell age in the fractionations of *E. coli* B/r. Helmstetter (Helmstetter, 1968; Helmstetter and Cooper, 1968) has previously shown that for this organism, growing in minimal-glycerol medium, initiation of DNA synthesis occurs at a cell age of about 0.8-0.9.

I have studied the rate of DNA synthesis in this organism, both in zonal separations and in synchronous cultures. Initiation of DNA replication was found to occur at about cell age 0.63. The reason for this difference from the data of Helmstetter is not known.

The potential for the synthesis of β-galactosidase, D-serine deaminase and tryptophanase synthesis was also studied. It is known that initiation of DNA replication occurs at map position 72' on the
E. coli chromosome (Masters, 1975). From this replicative origin
the chromosome is replicated bidirectionally (Masters and Broda, 1971;
et al. 1974). If we assume that the replication forks travel with equal
velocity, termination of replication would occur at map position 27',
diametrically opposite the origin.

The genes for β-galactosidase (lac), D-serine deaminase (dad)
and tryptophanase (tna), map at 9', 45', and 73' respectively (Taylor
and Trotter, 1972). Accordingly, the tna gene will be duplicated at
the time of initiation of replication, whilst the other two genes
(27' distant from the replicative origin) will be duplicated simulta-
neously at about midway to two-thirds through the cycle of DNA rep-
lication.

The potential for synthesis of these enzymes should double
at the same time as their structural genes during the replicative
cycle.

These expectations are shown to be approximately true in
both zonal fractionations and synchronous cultures (figure 26).

The zonal fractionations of E. coli B/r only resolved the
first two-thirds of the cell cycle. Events occurring in the latter
third of the cycle were therefore not open to investigation.

The inclusion of penicillin induced filaments in these
fractionations allowed a greater than two-fold increase in cell size
across the rotor to be observed. Conceivably, events in the latter
third of the cell cycle might thus be examined. In order to test this
possibility, the rate of DNA synthesis and the potential for trypto-
phanase synthesis, were studied in one such experiment (figure 22).
Although the increase in the rate of DNA synthesis and tryptophanase potential occurred at about the expected time, both then increased continuously. In the absence of a clear rate doubling it was not possible to determine a half-doubling time for the rate changes studied. Consequently, the cell ages at which these changes occurred were not derived.

3 Synthesis of RNA polymerase during the cell cycle:

The discontinuous synthesis of enzymes during the cell cycle has been discussed in the Introduction. Various explanations for this mode of gene expression have been advanced. Firstly, according to the Linear Reading Model, periodic gene expression is a consequence of an ordered, sequential, transcription of the genome. Secondly, according to the Oscillatory model of feedback repression, the continuous availability of the gene for transcription is modulated by a negative feedback control system. Thirdly, the periodic synthesis of enzyme during the cell cycle may be a metabolic perturbation introduced by the synchronising procedure.

Although enzyme synthesis is being considered here, rarely has it been measured. Rather, enzyme activity has been assayed and assumed to represent enzyme synthesis. In only a few cases have fluctuations in enzyme activity during the cell cycle been shown to be due to de novo enzyme synthesis (Mitchison and Creanor, 1969; Donachie, 1965). Whilst in some cases, fluctuations in enzyme activity have been shown to be due to the presence of small molecular weight effectors or potentiators of enzyme activity (Schmidt, 1974a; 1974b; Beck and Park, 1976). Only in two instances, both in mammalian cell studies, has enzyme protein, rather than enzyme activity, been measured; the
proteins were estimated immunologically (Martin, Tomkins and Granner, 1969; Klevecz, 1969).

Consequently, the synthesis of RNA polymerase, during the bacterial cell cycle, seemed an ideal system for study.

The β and β' subunits of RNA polymerase can be easily separated from other cell proteins, using SDS-polyacrylamide gel electrophoresis, by virtue of their high molecular weight, (155,000 and 165,000 respectively). Thus, using radioactively labelled preparations, β and β' can be estimated as a fraction of total labelled cell protein (Matsura, Hansen and Zeuthen, 1973). Using pulse labelled preparations, and chasing the label into completed polypeptide chains, allows the rate of β and β' synthesis to be estimated as a percentage of the total rate of protein synthesis. This percentage has been called the αₚ ratio (Matsura, Hansen and Zeuthen, 1973). Figure 27 shows the estimation of this value in glycerol grown cells of E. coli B/r. In practice, the ratio was expressed in terms of the tritium counts in the β and β' gel bands, over the total tritium counts applied to the gel. A more convenient value was the rate of enzyme synthesis per ml of culture; this is simply the product of αₚ and the total rate of protein synthesis per ml of culture, as determined by the incorporation of labelled precursor into TCA precipitable material.

Conveniently, β-galactosidase can be resolved using the same SDS-polyacrylamide gel system. Formerly, the rate of β-galactosidase synthesis had been expressed only in terms of enzyme activity following a short pulse induction i.e. the potential for enzyme synthesis.

The rate of β-galactosidase synthesis has been measured in exponential cultures, both enzymatically, and using the SDS-polyacrylamide gel electrophoresis method, above. Figure 28 shows the two methods to be comparable.
Thus a system exists whereby the rate of de novo enzyme synthesis can be directly measured for the $\beta$ and $\beta'$ subunits of RNA polymerase. For cells grown in the presence of $10^{-3}$ M IPTG, the rate of $\beta$-galactosidase synthesis can also be measured, thus providing a useful internal control.

The location of the rpo gene on the E. coli chromosome is known to be at 79' (Taylor and Trotter, 1972). There is also evidence which suggests that expression of the gene is autoregulated (Glass, Goman, Errington and Scaife, 1975).

Natzura et al (Natzura, Hanson and Zeuthen, 1973) studied the synthesis of $\beta$ and $\beta'$ during the cell cycle of glucose-grown E. coli. The cells were synchronised by the gradient selection technique, (Mitchison and Vincent, 1965). Neither the rate of $\beta$-galactosidase, nor the rate of DNA, synthesis were studied in these experiments. More importantly, only the $\omega_p$ ratio was reported. Although small fluctuations in this ratio were observed during synchronous growth, they were within the limits of experimental error. Thus it was concluded that $\beta$ and $\beta'$ were synthesised continuously during the cell cycle. In other words, there was no gene dosage effect upon the rate of enzyme synthesis, thereby demonstrating the close autogenous (Goldberger, 1974) control of gene expression.

However, the $\omega_p$ ratio is an extremely insensitive parameter - as indeed are all specific activity measurements - for use in cell cycle analysis. Throughout a cell cycle the ratio has a constantly increasing denominator, and so is insensitive to small periodic increases in the numerator, which, on average, is also increasing continuously. It is preferable to express data on a per ml basis (Mitchison, 1971) thus maintaining a constant denominator. Accordingly,
the results of Matsura et al are best regarded as being inconclusive.

I have examined the rate of β' and β-galactosidase synthesis during the cell cycle of *E. coli* B/r. Originally the investigation was carried out on pulse-chased labelled cells, fractionated in the zonal rotor. The rate of β' synthesis was found to increase continuously across the rotor, indicating no effect of gene dosage. However, the internal β-galactosidase control, was found to increase in the same manner (unpublished observations).

The synthesis of RNA polymerase was then investigated in synchronous cultures. The cultures were established by subculturing small cells from a zonal fractionation. The resulting large synchronous cultures had ample material in them to allow withdrawal of successive 10 ml aliquots for pulse-chasing and subsequent analysis by SDS-polyacrylamide gel electrophoresis. The data from one such experiment is shown in figure 29. Cell division and the DNA cycles are indicated. Most strikingly, there can be seen a substantial perturbation in the synthesis of β' and β-galactosidase, which was also evident, though to a lesser extent, in the rate of total protein synthesis. β-galactosidase synthesis is particularly easy to perturb (Donachie and Masters 1966; Masters, Kuempel and Pardee, 1964; Abbo and Pardee, 1960; this thesis, figure 24). The β-galactosidase synthesis, however, seemingly recovered from the perturbation, and showed a gradual rate doubling about two-thirds of the way through the DNA replicative cycle. The synthesis of the β' subunit perhaps does not recover from the perturbation. At all times however, the rpo gene was expressed, indicating an unrestricted availability of the gene for transcription. Arguably the absence of a rate doubling is a sign that expression of the gene is closely controlled, although the data is by no means unequivocal. The problem of metabolic perturbations in synchronised cultures was clearly demonstrated in this experiment.
Periodically synthesised proteins involved in cell division:
The evidence relating protein synthesis and cell division has been considered in the introduction. The requirement, of a period of protein synthesis, for cell division, occurring at or near termination of DNA replication, has been clearly demonstrated (Jones and Donachie, 1973; Dix and Helmstetter, 1973).

Also, certain temperature sensitive mutants have been studied (Inouye, 1969; Zusman, Inouye and Pardee, 1972) in which, at the restrictive temperature, the normal relationships between cell division and protein synthesis are disrupted.

The broad conclusions from these various studies were that a protein molecule(s), synthesised periodically only at termination of DNA replication, somehow effected subsequent cell division. Presumably the protein effector is highly labile, or alternatively, once synthesised, is somehow consumed in the division process. However, very few of the proposed models have taken into account our knowledge of gene expression during the cell cycle.

Donachie and Masters (Donachie and Masters, 1969) have considered the periodic appearance of a division protein in terms of a continuously expressed structural gene, which is autoregulated, and produces an unstable gene product. Presumably the basal level of division factor is too low to effect cell division. Doubling of the structural gene at termination would result in a burst of synthesis of gene product, sufficient to effect cell division. At the same time, not only would the unstable gene product become inactive, but so too would the expression of the structural gene be feedback repressed. This model would certainly produce a pronounced periodic burst of synthesis of division protein upon duplication of the structural gene located at
the replicative terminus.

An alternative model suggests that termination of DNA replication, by some novel means, actually induces expression of the genes for the termination proteins (Jones and Donachie, 1973; Donachie, Jones and Teather, 1973). There is, however, little concrete evidence for this sort of mechanism.

Certain proteolytic enzymes (protease, leucine aminopeptidase, and glycylglycine dipeptidase) have been studied during the cell cycle of *E. coli* K12 strains, (Nishi and Horose, 1966; Kogoma and Nishi, 1965). These enzymes were claimed to show a peak of specific activity at the time of cell division. Where enzyme per ml, rather than specific activity was plotted, then the familiar stepwise pattern of enzyme activity was seen. Thus the claimed "peak" enzymes, were really "step" enzymes. Furthermore, there was rarely more than a 50% difference in enzyme level between dividing and non-dividing cells. These small oscillations in enzyme activity cannot be considered appropriate to models of cell division, contrary to the authors claim. Indeed, there was no evidence to show that they were not artefacts of the synchronisation procedure (see Nishi and Horiuchi, 1966; this thesis, Introduction).

The linear reading model provides a hypothesis for the restricted, periodic expression of genes. However, this model is not considered to apply to prokaryotic systems (Mitchison, 1971).

Mitchison (Mitchison, 1976) has studied the synthesis of eighteen enzymes in *Schizosaccharomyces pombe*. Five enzymes (ATCase, OTCase, tryptophan synthetase, alcohol dehydrogenase, and homoserine dehydrogenase) formerly thought to be synthesised discontinuously, have now been shown to be synthesised continuously. By using suitable
controls, Mitchison has only convincingly shown the existence of one periodically synthesised enzyme: Thymidinemonophosphate kinase - an enzyme of DNA metabolism.

On the basis of such studies, it seems that periodically synthesised proteins (except in *Saccharomyces cerevisiae*) are something of a rarity. Although their existence has been readily invoked in models of cell division, they have rarely been demonstrated convincingly.

The reported, periodically synthesised, membrane proteins, implicated in the cell division process (Jones, 1974; Churchward and Holland, 1976) have been discussed in the Introduction. In apparent contradiction to these reports, Inouye and Pardee (Inouye and Pardee, 1970) claim to have found no periodically synthesised membrane proteins during the bacterial cell cycle. These workers resolved cells into size and age classes by sedimentation velocity centrifugation, using a sucrose density gradient in a centrifuge tube. This method lacks the resolution afforded by the zonal rotor: no details of the resolution were given by Inouye and Pardee.

Although the data concerning these periodically synthesised division proteins is somewhat equivocal, their existence is demanded by certain data (Jones and Donachie, 1973; Dix and Helmstetter, 1973). Thus, an attempt to clearly demonstrate such proteins was undertaken. The zonal rotor was used to resolve cells, labelled with tritiated leucine, into size classes. Three size classes were chosen (figure 31), one of which contained no dividing cells, another being greatly enriched with dividing cells. Each sample was mixed with $^{14}$C-leucine labelled exponential cells. All cells were in balanced growth at the time of labelling. From the double labelled samples, cell membranes
were prepared and analysed by SDS-polyacrylamide gel electrophoresis (10%/0.27% gel). The distribution of the two isotopes in the gels was analysed by slicing and solubilisation, followed by discrimination counting (Materials and Methods). There could be detected no protein synthesised in the exponential cells, which was not also synthesised in each discrete size class of cells from the zonal rotor. This finding was particularly significant, in that $^3$H labelled cells, containing 0% dividing cells, had synthesised all the protein species also synthesised in the exponential population, containing 15-20% dividing cells.

Lutkenhaus has improved the resolution of this sliced gel/double label analysis. He has used a 16%/0.09% gel system, and has analysed the distribution of isotope in such gels autoradiographically. Exponential cells were pulse labelled with $^{35}$S-methionine, and separated in the zonal rotor. Membranes were prepared from selected fractions and analysed on the above gel system. Plate IV is a photograph of an autoradiograph, of such a gel. Cells of all sizes from the zonal rotor were found to have synthesised identical membrane proteins during the pulse labelling. Lutkenhaus and Richardson (unpublished data) have duplicated this result using $^3$H-leucine labelled cells.

Two-dimensional gel electrophoresis has been used in this laboratory (Lutkenhaus and Richardson) in the search for any protein species synthesised only at one time during the cell cycle. Lutkenhaus and Richardson (unpublished data) found that, in zonal fractionations of *E. coli* B/r and *E. coli* K12 AB2497, and also in synchronous cultures of *E. coli* B/r, cells of all ages synthesised identical proteins. Plates V, VI and VII show three autoradiographs developed from some two-dimensional polyacrylamide gels. These were used to
analyse the rates of synthesis of cell proteins at various stages during synchronous growth of E. coli B/r. As far as can be discerned, cells of all ages had synthesised identical proteins during a \(^{35}\)S-methionine pulse.

Cooper has used the membrane-elution technique (Helmstetter and Cooper, 1968) to study the cell cycle of E. coli B/r. He examined cell protein on two-dimensional polyacrylamide gels, and concluded that all proteins were synthesised at all times during the cell cycle (pers. comm.).

Obviously, the periodic, or restricted, expression of genes during the E. coli cell cycle is an extremely rare occurrence. Most of the evidence so far collected in this laboratory points to this conclusion.

However, it is not possible to categorically exclude periodic protein synthesis from the E. coli cell cycle. So far though, the need for this mode of synthesis has only been convincingly described in models of cell division.

5

**Summary:**

A method for fractionating E. coli B/r into size and age classes has been described.

Large numbers of cells can be handled, permitting the establishment of large synchronous cultures. The advantage of these large cultures was exploited in the analysis of RNA polymerase synthesis during the cell cycle. Metabolic perturbations in the synchronous culture were, however, a serious problem.

The unperturbed cell cycle was studied in zonal rotor frac-
tions. The information concerning gene expression during the cell cycle was used to interpret findings which relate DNA replication, protein synthesis and cell division. Accordingly, several experiments were designed to seek for proteins synthesised only at one time in the cell cycle. Such proteins might, perhaps, be responsible for control of periodic cell cycle events - especially cell division. No such proteins were found, in contradiction to certain previous findings.

Periodically synthesised proteins cannot be fully discounted, however. It is likely that there are only a very few in E. coli. Indeed, it is difficult to build models of the temporal organisation of cellular events without invoking the existence of such proteins.
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7.b.i. Pretreatment and harvesting of cells:

As previously stated, cells were harvested, where possible, at a cell density of about $10^8$ cells per ml; an O.D. of 0.1. However, on a number of occasions cells were harvested at optical densities from 0.05 to 0.4. At higher cell densities slightly less effective cell size separations were achieved.

The dense cell suspension was usually briefly sonicated prior to application to the rotor. Suspensions which were not sonicated were again resolved less well in the subsequent fractionation.

These somewhat less effective cell separations were characterised by the marked presence of cells of all sizes in the lattermost fractions collected from the rotor. This was regarded as evidence for the clumping together of cells in the dense suspension. It seemed probable that any such clumps would migrate rapidly through the sucrose gradient, and might dissociate at any subsequent time during the fractionation procedure. Therefore, sonication of cell suspensions was routinely performed, and the number of cells loaded onto the gradient was kept to a workable minimum. In so doing, resolution of the larger cells was usually slightly improved.

The presence of small, newly divided cells in the latter fractions was reduced either by addition of 60 units per ml of benzylpenicillin to the cells upon harvesting, or by maintaining the cells at 0-4°C at all times subsequent
to harvesting, or both. These procedures also tended to improve the definition of the large cell containing fractions.

7.b.ii. Loading the gradient: application of the cell suspension: fractionation: collection of fractions:

and c. The sucrose gradient:

The schematic diagrams (figs. 3-8) relate to the application of a 5-15% sucrose gradient, linear with respect to rotor volume.

Several other gradient systems were used. Figures 3-8, however, still represent the basic procedures involved.

The gradient systems used were as follows:

i) A 5-15% sucrose gradient, linear with respect to rotor volume: the cells were loaded in 25-30mls of 3% sucrose and overlayed with 50-100mls of 1% sucrose: fractionation time was 20-40 minutes, and the gradient was displaced from the rotor centre by pumping dense sucrose (20-30%) to the rotor edge.

ii) A 5-15% sucrose gradient, linear with respect to rotor radius: all other conditions being as above.

iii) A 5-15% sucrose gradient, linear with respect to rotor volume: all other conditions being as above, except that the gradient was displaced from the rotor edge by pumping water to the rotor centre.

iv) A 3-8% sucrose gradient, linear with respect to rotor volume: the cells were loaded in 25-30mls of 1% sucrose, and overlayed with 50-100 ml of water: the fractionation time was 10-20 minutes, and the gradient was displaced
from the rotor centre by pumping dense sucrose (15-20%) to the rotor edge.

v) A 10-40% sucrose gradient, linear with respect to rotor volume: the cells were loaded in 25-30mls of 3% sucrose and overlayed with 50-100mls of 1% sucrose: fractionation time was greater than 40 minutes and the gradient was displaced from the rotor centre by pumping dense sucrose (40-50%) to the rotor edge.

The rate of displacement of the gradient from the rotor was varied in many experiments. Gradient was displaced slowly (about 50mls/minute) in most of the experiments already described. However, displacement rates of between 50 and 100mls/minute, or greater than 100mls/minute were investigated for some of the gradient systems described above.

7.d. Preparation of synchronous cultures:

A shallow gradient (3-8%), linear with respect to rotor volume, was used for fractionation of live cells at room temperature. The fractionation was achieved in 10-20 minutes. It was found that under these conditions, and by displacing the gradient at up to 100mls/minute, well resolved small cells could be obtained. The resolution of cells in the later fractions was, however, somewhat compromised. Such a gradient system was ideal for the rapid synchronisation of cells by subculturing the small cells thus obtained.
Separation of E. coli B/r into size classes:

The conditions used for the cell separation in figure 12 were claimed to be optimum for size resolution of E. coli B/r. Most of the fundamental experimentation on which this claim was based was not presented. The following figures and text outline certain conditions under which cell size separation was investigated.

Figure 12 shows the optimised separation of cells into size classes. The cells were harvested from balanced exponential culture at an O.D.₅₄₀ of 0.1. Cell division was blocked with 60 units/ml of benzylpenicillin, protein synthesis was blocked with 200 μg/ml of chloramphenicol, and the cells were rapidly chilled by pouring onto ice. All subsequent steps were performed at 0-4°C. The cells were concentrated by centrifugation, resuspended in about 25 mls of 3% sucrose, and briefly sonicated. Fractionation was achieved on a 5-15% sucrose gradient, linear with respect to rotor radius. After fractionation, the gradient was displaced from the rotor centre at about 50 mls/minute.

Identical fractionations have been performed in the 5-15% gradient, linear with respect to rotor volume. There were no significant, reproducible differences between the separations achieved on either gradient. However, the former proved to be more convenient in use.

Figure A1 shows the effect upon size separation of harvesting the exponential culture at O.D. 0.4, i.e. loading about 4 x 10¹¹ total cells on the gradient. All other conditions were identical to those described above for figure
Figure A1 shows that the cells were less well resolved than when less than $10^{11}$ total cells were applied to the rotor. Mean cell length has increased by only 55% across the rotor, compared with nearly 70% in figure 12. I have taken this to mean that cells clump together in the dense suspension applied to the rotor. Such clumps would migrate rapidly through the gradient and might dissociate at any later time during the fractionation procedure, thereby reducing the resolution of the separation method.

An analagous experiment was performed in which cells were harvested at O.D. 0.1, but the cell suspension was not sonicated prior to loading on the rotor. The data obtained from this experiment was essentially identical to that shown in figure A1.

The effect of cell fimbriae on the formation of cell clumps was investigated (fig. 13), but no clear distinction could be drawn between the size separations of fimbriate and afimbriate cells.

Figure 13A, however, indicates that penicillin, as a specific cell division block, improves resolution of the larger cells on a 5-15% sucrose gradient. It has been stated that the effect of penicillin on the efficiency of size resolution was somewhat variable. Chilling the cells rapidly by pouring onto ice at the time of harvesting, and carrying out all subsequent steps at 0-4°C, may be a sufficient condition to minimise cell division in the rotor.

Figure 18 shows that mean cell size increases by over 60% across the rotor in a separation omitting penicillin. No cell size distributions were derived for this particular experi-
ment. In most cases, though, it was preferred to include a specific division block, rather than rely solely on carrying out procedures at a reduced temperature.

Various gradient systems have been used for cell separation in the zonal rotor.

Figure A2 shows the effect of rapidly displacing (greater than 100mls/minute) the sucrose from the rotor. The gradient was 5-15%, linear with respect to rotor volume, all other conditions being identical to those described for figure 12. Mean cell length increased across the rotor by about 50%.

Figure A3 shows an identical experiment. In this case however, the gradient was displaced rapidly (greater than 100mls/minute) from the rotor edge by pumping water to the rotor centre.

Figure A4 shows cell fractionation on a 3-8% sucrose gradient, linear with respect to rotor volume. The separation was carried out at room temperature and no penicillin was included. The gradient was displaced from the rotor at 100 ml/minute.

This experiment has been performed at 0-4°C in the presence of penicillin, although the fast pumping rate was still used. There was only a very minor improvement in size resolution achieved. This gradient system has not been investigated using a slow rate of gradient displacement from the rotor.

Figure A5 shows fractionation of cells on a 10-40% sucrose gradient, linear with respect to rotor volume, which
was then displaced rapidly from the rotor.

It may seem that some of the optimising conditions described in figure 12 produced only a marginal or even insignificant improvement upon the data presented here. These experimental conditions were however, developed empirically, and taken together, as in figure 12, helped to provide consistent, reproducible and experimentally useful cell separations.

The range of conditions tested, although by no means exhaustive, give credit to the following notions:-

i) That penicillin, included as a specific block to cell division, together with performing all operations at 0-4°C, reduces contamination of large cell containing fractions with newly divided cells.

ii) That any tendency for the formation of cell clumps to impair the resolution of the larger cells can be minimised by loading as few cells on the rotor as is conveniently possible, (in practice about $10^{11}$), and by briefly sonicating the suspension prior to application to the gradient.

iii) That a 5-15% sucrose gradient(either linear with respect to rotor volume or rotor radius) is adequately stable to permit the best possible separation of cells in the least possible time.

iv) That there is turbulence and viscous drag upon displacement of the gradient from the rotor. The actual separations in a 5-15% gradient must be identical, or nearly so. Yet the subsequent mode of collection of fractions seemingly disrupts the gradient in some way to produce apparently
variable separations. For example, rapid displacement of the cells from the rotor reduces the effectiveness of separation, particularly in an insufficiently stable gradient (figure A4) and when reversing the direction of displacement (figure A3).

Although these notions have by no means been rigorously statistically proven, however, they have provided a workable system of cell separation which has been adequate for cell cycle studies.
Appendix to Discussion, section 2:

2. Fractionation of cells into age classes:

The relationship between cell size and cell age is central to this thesis.

This relationship has been examined for *Saccharomyces cerevisiae* (Sebastian, Carter and Halvorson, 1971). They suggest that "if fractions from the zonal rotor contain cell populations at different stages of the cell cycle, it should be possible to remove different fractions from the zonal rotor and successfully initiate synchronous growth." I have demonstrated this for *E.coli* B/r, by removal of small cells from zonal fractionations, from which synchronous growth ensued upon subculture. Cell division occurred after one generation time indicating that the smallest cells in the zonal rotor were also the youngest.

Sogin, Carter and Halvorson, (1974), have compared the size distribution of successive zonal fractions with the size distributions of yeast cells of different ages taken from a synchronous population. They showed that the distribution of cell sizes at successive stages of the cell cycle were comparable with the distribution of cell sizes in successive fractions from the zonal rotor. I have not performed this comparison myself for *E.coli* B/r. However, I have produced size distributions for cells in successive fractions across the zonal rotor. Helmstetter and Cummings (1963) have produced synchronous cultures of *E.coli* B/r, in which successive cell size distributions have been studied. These also represent age distributions in the selection system used by
Helmetstetter and Cummings. They are comparable to the size distributions obtained for successive zonal fractions of *E. coli* B/r in this thesis.

I have also produced data for the increase of mean cell size across the zonal rotor, expressed as an O.D. per cell ratio. Figure A6 shows data from a synchronous culture in which this O.D. per cell ratio was also determined. The dotted line indicates the hypothetical ideal where cell division is instantaneous and perfectly synchronised. Note that the youngest cells have a size of approximately 70 units. This corresponds with the smallest cells derived from zonal fractionations. The change in the rate of DNA synthesis in this synchronous culture occurs at a cell age of 0.6, and at a cell size of 115 volume units. In figure 20, this change in rate of DNA synthesis was found to occur at a cell size of 115 units, which was calculated to represent a cell age of 0.65. Therefore, stage in the cell cycle relates closely to cell size in the zonal rotor.

Cell cycle age markers represent a further correlation between cell size and cell age. Helmstetter (1968) eluted cells from a membrane filter according to their stage in the cell cycle. He has shown that the changes in the rates of DNA and tryptophanase synthesis occur in older cells, whilst the change in rate of β-galactosidase synthesis occurs in younger cells. I have shown the former two rate changes to occur in larger cells, whilst the latter rate change occurs in smaller cells.
Mitchison, 1971), the cell age distribution is defined by

\[ y = 2^{(1 - x)} \]

or

\[ \log_2 y = (1 - x)\log_2 2 \]

where \( y \) (varying from 1 to 2) is the relative number of cells at cycle stage \( x \) (varying from 0 to 1).

This ideal relationship is shown graphically in figure A7. The dispersion of interdivision times among the members of an exponential population alters this ideal curve. The real relationship between relative cell number, and stage in the cell cycle, is reflected in the distribution of cell numbers across the zonal rotor (figure 17). Thus showing that cells are distributed across the zonal rotor according to their stage in the cell cycle as well as their size.

These several criteria, taken together, indicate a close correlation between cell size and cell age across the zonal rotor.
E. coli B/r was grown at 37°C in 1 litre of glycerol-minimal medium for greater than 15 generations. At O.D. 540 of 0.4, the cells were treated with 60 units per ml of benzylpenicillin and 200 μgm per ml of chloramphenicol, poured rapidly onto ice, and pelleted by centrifugation. The cell pellet was resuspended in 25-30 mls of ice-cold 3% sucrose containing penicillin and chloramphenicol at the above concentrations. The suspension was briefly sonicated, and applied to a pre-cooled 5-15% sucrose gradient, linear with respect to rotor radius, in the zonal rotor at 4°C. The cells were fractionated for c.30 minutes, and fractions were collected on ice from the rotor by displacement of the gradient from the rotor centre with dense sucrose. A pumping rate of 50 mls/minute was used.

Cell size distributions were measured photomicrographically. Fraction numbers (fn) and mean cell lengths (L) are shown.


**FIGURE A1:**

- **Mean Cell Length vs. Fraction Number**
- **Cell Length in Microns**
- Histograms showing the distribution of cell lengths for different fractions.
Figure A2:

E. coli B/r was grown at 37°C in 1 litre of glycerol minimal medium for greater than 15 generations. At O.D. 540 of 0.1 the cells were treated with 60 units per ml of benzylpenicillin and 200 µg/ml of chloramphenicol, poured rapidly onto ice and pelleted by centrifugation. The cell pellet was resuspended in 25-30 mls of ice-cold 5% sucrose containing penicillin and chloramphenicol at the above concentrations. The suspension was briefly sonicated and applied to a 5-15% sucrose gradient, linear with respect to rotor volume, in the zonal rotor at 4°C. The cells were fractionated for c.30 minutes, and fractions were collected on ice from the rotor by displacement of the gradient from the rotor centre with dense sucrose. A pumping rate of greater than 100 mls/minute was used.

Cell size distributions were measured photomicrographically. Fraction numbers (fn) and mean cell lengths (L) are shown.
Figure A3:

This experiment was identical to that described in figure A2, except that the gradient was displaced from the rotor edge by pumping water to the rotor centre.

Cell lengths were determined photomicrographically. The fraction numbers (fn) and mean cell lengths (L) are shown.
FIGURE A3:

1. Mean cell length vs. fraction number.
2. Distribution of cell length in microns for different fraction numbers.

- fn 1: Mean length 1.55 μm
- fn 6: Mean length 1.7 μm
- fn 11: Mean length 2.2 μm
- fn 20: Mean length 2.2 μm
- fn 25: Mean length 2.2 μm

Cell length in microns for each fraction number.
E. coli B/r was grown at 37°C in 1 litre of glycerol minimal medium for greater than 15 generations. At O.D.\textsubscript{540} of 0.1 the cells were pelleted by centrifugation at room temperature. The cells were resuspended in 25-30mI of 1% sucrose. The suspension was briefly sonicated and applied to a 3-8% sucrose gradient, linear with respect to rotor volume, in the zonal rotor at room temperature. The cells were fractionated for c.15 minutes, and the fractions were collected from the rotor by displacement of the gradient from the rotor centre with dense sucrose. A pumping rate of 100 mls/minute was used.

Cell size distributions were measured photomicrographically. The fraction numbers (fn) and mean cell lengths (\overline{L}) are shown.
FIGURE A4:
E. coli B/r was grown at 37°C in 1 litre of glycerol minimal medium for greater than 15 generations. At O.D. 540 of 0.1 the cells were treated with benzylpenicillin at 60 units per ml and chloramphenicol at 200 µgm/ml, poured rapidly onto ice and pelleted by centrifugation. The cell pellet was resuspended in 25-30 mls of 3% sucrose (ice-cold) containing penicillin and chloramphenicol at the above concentrations. The suspension was briefly sonicated and applied to a pre-cooled 10-40% sucrose gradient, linear with respect to rotor volume, in the zonal rotor at 4°C. The cells were fractionated for greater than 40 minutes, and fractions were collected on ice by displacement of the gradient from the rotor centre with dense sucrose. A pumping rate of greater than 100 mls/minute was used.

Cell size distributions were measured photomicrographically. Fraction numbers (fn) and mean cell lengths (L) are shown.
**Figure 15**

- **Mean Cell Length**
  - Graph showing the mean cell length in microns against fraction number.
  - Fraction numbers range from 1 to 25.
  - Cell lengths range from 1.5 μm to 2.7 μm.

- **Cell Length Distribution**
  - Histograms for different fraction numbers (fn 1, fn 5, fn 10, fn 15, fn 20, fn 25).
  - Each histogram shows the distribution of cell lengths in microns.
  - Mean cell lengths for each fraction are indicated (e.g., fn 1: 1.5 μm, fn 20: 2.4 μm).
**Figure A6:**

_E. coli_ B/r was separated in the zonal rotor as described in figure A4.

Small cells from this fractionation were used to establish a synchronous culture. Cell numbers per ml, O.D. \(_{540}\), and the rate of DNA synthesis were measured as previously described. Cell size throughout a synchronous division was estimated in terms of O.D. per \(10^{11}\) cells.

The time of cell division, and the change in rate of DNA synthesis are indicated, together with the cell ages at which these events occurred.

A) Mean cell size; O.D/\(10^{11}\) cells. The bold line represents the values derived from the graphs below; the broken line represents the hypothetical ideal where cell division is instantaneous and perfectly synchronised.

B) O.D. \(_{540}\) nm

C) Cells per ml \(\times 10^{-7}\)
FIGURE A6:
Figure A7:

Relative frequency \((y)\) of cells at stage of the cell cycle \((x)\) for cells from an asynchronous exponential phase culture. The bold line is an ideal curve, based on the relationship \(y = 2^{(1-x)}\) (from Mitchison, 1971).

The broken line represents a real age distribution allowing for the variation of interdivision times of the members of the exponential population. (This is a schematic representation and is not based on any data or precise mathematical description).
FIGURE A7