STUDIES ON THE RELATIONSHIP BETWEEN CHROMOSOME REPLICATION AND CELL DIVISION IN ESCHERICHIA COLI

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by

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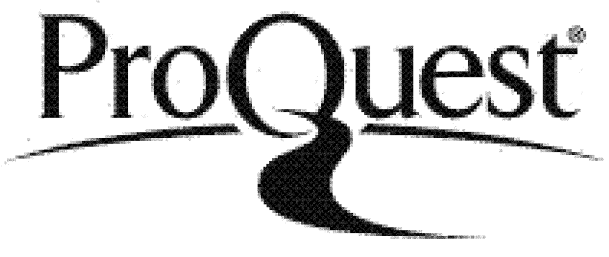

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1. INTRODUCTION

1.I. ANALYSIS OF THE BACTERIAL CELL CYCLE

The life cycle of a bacterial cell consists of the collected sequence of events occurring within the cell from the time of its inception, as a result of division of a parental cell, until its own division. Within a population of bacterial cells growing exponentially in an unlimited supply of nutrients the average duration of the interdivision period is equivalent to the time taken for each extensive parameter of the culture to double, and is known as the Generation Time τ. During this period, which is characteristic for the set of growth conditions, every cell increases the amount of each constituent so that at division it has, almost exactly, twice the size and composition that it inherited at birth. At division the cellular components are partitioned between the two daughter cells such that they are essentially indistinguishable from each other. Thus the processes which take place during the life cycle of a bacterial cell do so under conditions of regulation and co-ordination so that the integrity of the cell species is maintained.

Our present understanding of the events of the bacterial cell cycle and their relationship to each other has come from data obtained by three experimental approaches, these being observations on individual cells, measurements at different cell ages and measurements on steady-state exponential cultures. The results obtained from these

provide somewhat different but complementary information about the cell cycle. Each too experiences its own peculiar limitations.

a) Observations on individual cells

Microscopical examination of bacterial populations can be used to provide two types of information. Firstly it can provide a morphological description of the events occurring during the division cycle, and give some idea as to their temporal relationship. For instance, time lapse photomicroscopy studies of growing and dividing cells have shown that cells of Escherichia coli grow by extension in length (Adler & Hardigree, 1964; Donachie & Begg, 1970) and that cell division is preceded by division and segregation of the nuclear material (Mason & Powelson, 1956). By the use of autoradiography these observations can be extended to examine the way in which specific cell components are segregated to the progeny cells at division (e.g. Lin, Hirota & Jacob, 1971; Tubergen & Setlow, 1961). Secondly the technique can be used to investigate the amount of variance in an exponential population. Many workers have attempted to analyse the spread of the distribution of individual cell generation times and the nature of the correlations, if any, between related cells in a random exponential population (Kubitschek, 1962; Painter & Marr, 1968; Powell, 1956; Schaechter, Williamson, Hood & Koch, 1962). Although the results are somewhat conflicting in detail, it seems generally agreed that there is a positive

correlation between the generation times of sister cells and a negative correlation between mother and daughter cells. If a cell is late in its own division then the generation times of its progeny will be shortened but remain approximately equal. This suggests that bacteria are in someway able to regulate the frequency of division so that there is on average, one act of cell division for each doubling in cell mass. Schaechter et al. (1962) have reported that the spread of the distribution in cell sizes at division is less than the variance in cell age and have concluded that division occurs at the attainment of a critical size. It would appear also that the distribution of generation times in populations growing on solid media is skewed towards longer times (Powell, 1956). Kubitschek (1962) has suggested that this might be a reflection of a normal distribution of the specific growth rates of the individual cells. Such a skewed distribution may be an important factor in determining the nature of synchronous cultures generated from surface-bound populations (Helmstetter & Cummings, 1964).

A major criticism against all analyses of this type is that the measurements are generally made at the limits of resolution of the technique employed. The difficulties in making accurate measurements and observations under such conditions may introduce considerable bias into the results.

b) Measurements at different cell ages

The development of techniques for the production of synchronously growing cultures of bacteria has enabled investigations to be made into the temporal relationships between events of the cell cycle. Providing that there is no variation in the size of the intracellular precursor pool with cell age, pulse-labelling studies can provide useful data about the nature of macromolecular synthesis. If any fluctuation in the rate of synthesis is repeated periodically over several generations then it is generally indicative of the action of a regulatory mechanism. An example of this type of analysis is the work of Clark and Maaløe (1967). They found that in glucose grown synchronous cultures of E.coli B/r the rate of DNA synthesis, measured by thymidine incorporation, undergoes an abrupt doubling in mid cell cycle. This stimulation in synthetic activity is due to initiation of new rounds of chromosome replication.

There is an alternative method by which the duration and rate of macromolecule synthesis can be measured as a function of cell age. It depends upon the ability to fractionate cells in exponential populations according to their various ages, or sizes, (Helmstetter, 1967; Mitchison & Vincent, 1965). By pulse-labelling an exponential culture with a radioactive precursor, age-fractionating the cells, and scoring the age classes which have incorporated radioactive material to varying degrees, an assessment can be made of the relative rate of synthesis of the macromolecule throughout the cell cycle.

Helmstetter, (1967) has used the ability of E.coli B/r, to bind to and grow on membrane filters, as a means of age-fractionating exponential populations. By pulse-labelling with radioactive thymidine he has been able to measure the rate of DNA synthesis as a function of cell age over a range of growth rates. Similarly the rate of DNA synthesis at various cell ages has been measured in populations sorted into size classes, either by sucrose gradient centrifugation (Gudas & Pardee, 1974; Kubitschek, Bendigkeit & Loken, 1967) or visually (Chai & Lark, 1970). In these studies it is generally assumed that cell size relates to cell age. This method, age or size fractionation, has an advantage in that the pulse-labelling is applied only once and to a steady-state exponential population. All the cells experience the pulse treatment under identical conditions which is not the case in the serial pulse-labelling studies generally used with synchronous cultures.

By the application of inhibitors of specific processes at various times during the growth cycle of a synchronous culture the requirement of one process for another can be examined. This method has enabled several workers (Clark, 1968b; Helmstetter & Pierucci, 1968) to conclude that completion a round of chromosome replication is required in order for division to occur. Although experiments of this nature can demonstrate an absolute requirement of one process for another, they do not provide immediate information about "timing-relationships" between the two processes, or events. An indication that one event is timed from another can best be obtained from experiments in which

the cell age of the triggering event can be varied. If such a temporal relationship does exist it will be evidenced by a similar change in the time of occurrence of the second event so that the interval between the two is unaltered. Perhaps the best example of this is the experiments of Helmstetter and Cooper (1968) who found that in E.coli B/r over a three-fold range of generation times the period between initiation of a round of chromosome replication and the subsequent cell division remained constant at 63 minutes.

There are two alternative principles underlying the methods for the production of synchronously dividing populations of bacteria. The first is that application of various treatments, e.g. heat treatment, starvation or addition of inhibitors, to an asynchronous population will cause all the cells eventually to become arrested at a particular stage of their division cycle so that when the regime is abolished the cells grow in a synchronous manner, (Cutler & Evans, 1966; Jones & Donachie, 1973). Such a synchrony is termed "induced" because it is imposed upon the cells, and often results in some sort of metabolic perturbation to the culture. Furthermore the particular method employed may not synchronise all cell processes. For example neither of the treatments used to align the bacterial chromosomes in the studies of Abe (1974) and Jones & Donachie (1973) cause an alignment of cell mass. Thus great care must be taken in the interpretation of the results obtained from experiments using these synchronised populations. The alternative, and perhaps preferable, approach to obtaining synchronous cultures is based upon the

observation that within any random exponential population there are sub-populations of cells growing in phase. Thus by preferential selection of these cells a synchronous population will be obtained with minimum perturbation to the culture. The selection can be on the basis of age (Helmstetter & Cummings, 1964) or size (Mitchison & Vincent, 1965). These techniques suffer from the disadvantage that the amount of material is substantially reduced because only a small proportion of the original population is used.

As there is considerable variation in individual generation times in any bacterial culture it is impossible to produce a culture which will maintain a high degree of synchrony over several generations of growth. Cultures which do show a prolonged periodicity can be produced but are dependent upon repeated treatments applied to the culture to maintain the "phasing" (Goodwin, 1969). The same criticisms apply for these cultures as for those in which synchrony is "induced". Because of the relatively short life time of synchronous cultures experiments are generally conducted to use the first and second generations of growth. In such circumstances it is important to ensure that the disturbance caused to the cells' physiological state during the synchronisation procedure is minimal, as the period of the experiment would be that during which the cells were readjusting to their normal growth pattern. It has been suggested (Kubitschek, 1970) that even the membrane-binding technique (Helmstetter & Cummings, 1964) for synchronising E.coli B/r may be suspect in this respect because the cells divide faster when bound to the solid

surface (Helmstetter, 1967). Thus they may be experiencing some sort of physiological "shift-up", from which they will have to readjust when regrown in liquid medium. Additional complications may arise if the method of producing the synchronous population introduces any bias into the frequency distribution of cells at particular ages or sizes. For instance although cells fractionated by sucrose gradient centrifugation may all have the same size they will not all be of the same age. The rather long period observed before the first synchronous burst of division in such cultures (Gudas & Pardee, 1974) may indicate that the small cell fraction largely consists of progeny from cells which divided prematurely in the previous cycle (Pritchard, 1975).

Finally it should be noted that although synchronous cultures can be used to provide information about temporal positions during the cell cycle the accuracy with which these measurements can be made is dependent upon the variance in the population. If the degree of synchrony is low, as evidenced by a large spread relative to the generation time, then distortions might arise in the measurement of the duration of processes occupying only a small proportion of the division cycle. This could be a contributory factor for the present controversy over the length of the replication period in E.coli under conditions of slow growth (Pritchard, 1975).

c) Measurements on steady-state exponential cultures

Growth of a bacterial culture has been defined by Campbell (1957) as being "balanced" if "during a time interval every extensive property of the growing system increases by the same factor". According to this definition a culture is in balanced growth if the ratio between two, or more, measured properties remains constant during the growth of the culture. Recently Zaritsky and Pritchard (1973) have described circumstances where this definition does not adequately describe the exponential growth of a culture. They have found that when thymineless mutants of E.coli are grown in low concentrations of thymine at fast growth rates the rate of increase in particle number, although exponential, is slower than the rate of increase in culture mass. The result is that average cell size increases continuously. However, the rate of DNA synthesis exactly parallels that of culture mass so that the DNA/mass ratio remains constant. These studies show that the normal state of exponential growth of a culture may not always be a balanced state. Perhaps a broader definition, suggested by Pritchard and Zaritsky (1970; Zaritsky & Pritchard, 1973), is that growth can be said to be in a normal state if the relationship between measured parameters is maintained over several generations of growth.

In any balanced exponential population there is a frequency distribution of cell ages such that the youngest cells are present in the greatest numbers and the oldest in the fewest, (Powell, 1956; Sueoka & Yoshikawa, 1965). For

an ideal population the relative frequency f (x) of cells at age x is given by the equation

$$f(x) = k \ln 2.2^{1-x} \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where x lies between 0 and 1.

It follows then that if events of the cell cycle have a temporal relationship those which occur earliest will be evidenced by their greatest frequency in the population. Thus by measuring the relative frequencies of suitable markers the events of the cell cycle can be ordered temporally. In this way the relative dosage of genes on the bacterial chromosome can be used to determine the sequence of replication. This technique was first utilised by Yoshikawa and Sueoka (1963) who used a transformation system to assay the relative dosages of genes in exponential populations of Bacillus subtilis. They demonstrated that replication takes place in an ordered sequence from a unique origin. This analysis has now been applied to E.coli (Bird, Louarn, Martuscelli & Caro, 1972; Masters & Broda, 1971) and by comparison of the marker frequencies with the linkage map it has been concluded that replication proceeds in a bidirectional manner. Conclusions about the mode of replication, whether uni-or bidirectional, could not be drawn from the experiments on B.subtilis because of the lack of a suitable method for long distance genetic mapping.

Knowledge of the age distribution enables calculations of the duration of cellular processes to be made from observation of the proportion of the population exhibiting

results of the process under study. For example, pulse-labelling of exponential cultures of E.coli with radioactive thymine and subsequent autoradiographic analysis of the fraction of cells showing no incorporation has been used to estimate the duration of the replication period (e.g. see Lark, 1966). Experiments of this nature should be treated with caution as it is necessary to have independent evidence, perhaps from synchronous culture studies, of the time in the cell cycle at which any gap occurs between periods of synthesis. Because of the difference in the relative number of cells at each stage of the cell cycle a different result will be obtained if it is assumed that the gap in synthetic activity occurs early in the cycle rather than late. In a similar way the fraction of cells showing two distinct nuclei can be used to estimate the cell age at which nuclear segregation occurs (Sargent, 1974).

The demonstration that bacterial cultures can exist in a great many distinct physiological states of balanced growth was made by Schaechter and co-workers (Schaechter, Maaløe & Kjeldgaard, 1958). They found that each steady-state of growth, which is dependent solely upon the growth rate afforded by the medium at a particular temperature, is characterised by a unique cell size and macromolecular composition. Analysis of the trend of changes in steady-state cell composition over a wide range of growth conditions has revealed that many properties such as cell mass and DNA content are functions of the growth rate of the culture. It is argued (Maaløe & Kjeldgaard, 1966)

that this is indicative of precise regulatory mechanisms operating on all cellular processes to maintain the cell in its appropriate dynamic equilibrium for each particular growth condition. Measurements of these parameters do not immediately give information about the patterns of synthesis in individual cells, they provide mean values for a population which consists of cells at all stages of the division cycle. However, if taken over a period encompassing several generations of growth the measurements become subject only to the random variations in sampling and can therefore provide a very accurate description of the growth state of the culture. These steady-state measurements can be used to quantitate changes in the duration of processes occurring during the cell cycle. Studies (Cooper & Helmstetter, 1968) using other techniques have shown that the period C taken for a complete round of chromosome replication and the interval D between termination of the round of replication and cell division are both constant over a three-fold range of growth rates. Several workers (Cooper & Helmstetter, 1968; Donachie, 1968; Pritchard, Barth & Collins, 1969; Pritchard & Zaritsky, 1970) have been able to derive theoretical relationships which express properties, such as average cell mass ($\bar{M}$) and DNA content ($\bar{G}$) and the DNA/mass ratio ($\bar{G}/\bar{M}$), of an exponential culture in terms of these cell cycle parameters C, D and τ.

$$\bar{G} = \frac{\tau}{C.\ln 2}\left(2^{(C+D)/\tau} - 2^{D/\tau}\right) \quad \ldots\ldots (2)$$

$$\bar{M} = k.2^{(C+D)/\tau} \quad \ldots\ldots (3)$$

$$\bar{G}/\bar{M} = \frac{\tau}{k.C.\ln 2}\left(1 - 2^{-C/\tau}\right) \quad \ldots\ldots (4)$$

where k is the cell mass at which initiation of a round of replication takes place, (Donachie, 1968; Pritchard et al. 1969). The agreement between the predicted variation in $\bar{M}$, $\bar{G}$ and $\bar{G}/\bar{M}$ for changes in growth rate, with that observed experimentally (Cooper & Helmstetter, 1968; Helmstetter, Cooper, Pierucci & Revelas, 1968; Pritchard, 1974), substantiates the validity of the conclusion that the initiation mass k is independent of growth rate. If these equations and their underlying assumptions are correct then measurements of changes in the steady-state composition, of cultures in different growth conditions, can be used to estimate the magnitude of any changes in C, D and τ. For example, Pritchard and Zaritsky (1970) have demonstrated that changes in the steady-state DNA/mass ratio can be used to accurately quantitate changes in the length of the replication period C. These theoretical relationships represent the ideal situation and do not take into account the natural variance within an exponential population. Painter (1974) has recently analysed the effects of statistical variation in the length of cell cycle parameters on the results obtained from this type of

analysis. He finds that the simple equations are in many cases a "good approximation".

The response of an exponential culture following treatments which perturb its balanced state of growth can give useful information about the nature of control mechanisms operating on cellular processes. It is a well documented observation (Kjeldgaard, Maaløe & Schaechter, 1958) that when exponential cultures are transferred to media supporting a different growth rate the rates of increase of various cell components do not all change together. The pre-shift rate of synthesis of various macromolecules is maintained for different lengths of time. In fact the dissociation of these processes during such a transition is the cause of the differences in the steady-state composition at the two growth rates. It is suggested (Kjeldgaard et al. 1958; see also Maaløe & Kjeldgaard, 1966) that this "pre-shift rate maintenance" is evidence of precise rate controlling mechanisms operating on the separate processes. In fact the extent of this delay following a transition can provide an estimate of the temporal relationship involved in the control mechanism. For if the occurrence of one cell cycle event commits a cell to another after a fixed time, conditions which alter the frequency of the former, e.g. acceleration following a nutritional shift up, will, cause a similar alteration in the rate of the latter. However, there will be a delay between the two changes in rate, equal to the length of the period between the two events. Thus the 70 minute delay during which cell division continues at the pre-shift rate

following a shift-up (Kjeldgaard et al. 1958) is a reflection of the control mechanism determining the frequency of cell division. The significance of this result can be appreciated from the knowledge that the period between initiation of a round of chromosome replication, which occurs whenever the cell reaches a critical size (Donachie, 1968), and the subsequent cell division is invariant at 63 minutes (Cooper & Helmstetter, 1968).

In an analogous manner treatments which inhibit the starting of specific processes can be used to estimate the duration and rate of the process. The increment in DNA (ΔG) following starvation of a culture for a required amino acid, a treatment which blocks further acts of initiation of replication (Maaløe & Hanawalt, 1961), can be used to estimate the length of the replication period C (Pritchard & Zaritsky, 1970), and the number of replicating units (Donachie, 1969; Sueoka & Yoshikawa, 1965) from the equation:

$$\Delta G = \left[\frac{n.2^n.\ln 2}{2^n - 1} - 1 \right] \times 100 \quad \ldots\ldots (5)$$

where n is equivalent to C/τ.

Observation of the period for which cell division continues after application of DNA synthesis inhibitors to exponential cultures has led to the suggestion (Clark, 1968a,b; Helmstetter & Pierucci, 1968) that completion of a round of replication is a necessary condition for division to occur.

1.II. CHROMOSOME REPLICATION IN BACTERIA

The chromosome of E.coli consists of a single endless molecule of double-stranded DNA (Cairns, 1963b; Jacob & Wollman, 1961). It is replicated sequentially in a semi-conservative manner from a unique origin (Cairns, 1963a; Lark, Repko & Hoffman, 1963; Meselson & Stahl, 1958). Autoradiographs of chromosomes released from alignment at initiation in low specific activity radioactive thymine and later pulsed with high specific activity material, show linear low density grain tracks which are bounded by more dense regions (Prescott & Kuempel, 1975). This pattern of incorporation is consistent with the symmetrical movement of two replication forks away from a common origin. Further biochemical evidence in support of a bidirectional mode of replication has been provided by McKenna and Masters (1972) who have analysed the susceptibility to ultraviolet radiation of newly replicated DNA containing bromouracil incorporated in the region of the replication origin. Their results show that the newly made DNA can be broken into two equal sized portions by the action of the radiation, thereby indicating that the bromouracil substituted region must lie between the growing points. Studies in which the gradient in frequencies of various chromosomal loci has been measured, either by P1 transduction (Masters & Broda, 1971) or by DNA hybridisation against resident prophage (Bird et al. 1972), give results compatible with a bidirectional mode of replication from an origin, located in the region minute

65 to minute 75 of the genetic map, to a terminus diammetrically opposite.

Less is known about the completion of the replication process. The symmetrical nature of the marker frequency analysis curves (Bird et al. 1972) together with auto-radiographic indications that most, if not all, terminations occur with both replication forks reaching the terminus at the same time (Kuempel, Maglothin & Prescott, 1973), lead to the conclusion that replication probably ceases when, and where, the two oppositely travelling forks meet. However, it does not exclude the possibility that the terminus of replication is also denoted by a unique region of the DNA molecule. This may be resolved by the use of "Integratively Suppressed" strains in which the control and origin of chromosome replication appear to be determined by an integrated sex factor (Nishimura, Caro, Berg & Hirota, 1971; Tresguerres, Nandadasa & Pritchard, 1975). Bidirectional replication of a circular chromosome has also been demonstrated in the related enteric organism Salmonella typhimurium (Fujisawa & Eisenstark, 1973) and in the Gram-positive bacterium B.subtilis (Gyurasits & Wake, 1973; Wake, 1974).

In contradiction to the previous claims for an increasing rate of DNA synthesis during the cell cyle (Abbo & Pardee, 1960) Maaløe (1961) argued that the rate of replication at a growing point was probably constant and independent of growth rate so that the overall rate of DNA synthesis in an exponential culture is determined by control of the frequency at which new rounds of

replication are started. He had shown (Maaløe & Hanawalt, 1961) that the fraction of an exponential population which is immune to thymineless death, the rapid loss in viability observed when thymineless strains are deprived of thymine (Barner & Cohen, 1954), could be increased by starving the cells of a required amino acid before removing the thymine. Moreover it was also shown (Hanawalt, Maaløe, Cummings & Schaechter, 1961) that the size of the immune fraction was equivalent to the number of cells in the population not actively engaged in DNA synthesis. It was therefore postulated that during inhibition of protein synthesis by amino acid starvation bacterial cells complete rounds of chromosomal replication which were already in progress but are unable to initiate new ones until the required amino acids are restored. In confirmation of this hypothesis, Lark et al. (1963) produced data to show that no portion of the DNA replicated during amino acid starvation is replicated a second time until the deprivation is ended. The first evidence in direct confirmation that the rate of replication is constant at a growing point was provided by the uniform density grain tracks in the autoradiographs of radioactively labelled chromosomes published by Cairns (1963a).

One consequence arising from the restrictions of a constant chromosome replication time, implicit in the hypothesis of a constant rate of replication, is that it becomes necessary for cells to engage in multifork replication at fast growth rates in order to duplicate their DNA content in the short generation time. That this

is the case was first demonstrated by Oishi, Yoshikawa and Sueoka (1964) who observed an increased gradient in transforming-marker frequencies during outgrowth of B.subtilis spores in rich media. Multiple fork, or "dichotomous" replication was also described by Pritchard and Lark (1964). They suggested that the increased rate of DNA synthesis following restoration of thymine to a starved culture is due to synthesis at extra growing points added to the chromosome during the starvation, in addition to those already present when thymine was removed. They concluded that these "premature initiations" were due to continuing growth in the absence of thymine, rather than an effect of the base itself. These observations gave support to the hypothesis that replication is regulated at the level of initiation and indicated that control is directly or indirectly mediated by cell growth. The converse of dichotomy, namely that in slowly growing cultures there is a portion of the cell cycle during which the cell does not synthesise DNA, had been apparent in the measurements of fractions of cell populations immune to thymineless death (Maaløe, 1961) and more directly demonstrated by the autoradiography studies of Schaechter, Bentzon and Maaløe (1959) and Lark (1966).

The development of a reliable selective method for the generation of synchronous cultures of E.coli (Helmstetter & Cummings, 1964) enabled studies to be made of the rate of DNA synthesis during the division cycle. By pulse-labelling a glucose grown synchronous culture of E.coli B/r with radioactive thymidine Clark and Maaløe

(1967) found that the rate of replication is constant but doubles abruptly in mid cell cycle. From measurements of the amount of DNA synthesised in the presence of the protein synthesis inhibitor chloramphenicol, they concluded that the doubling in the rate of synthesis reflects an increase in the number of replication forks. Thus the rate of DNA synthesis at each fork is constant. Cooper and Helmstetter extended this type of analysis in a series of experiments in which they measured the rate of DNA synthesis as a function of cell age over a wide range of growth rates (Helmstetter, 1967; Helmstetter & Cooper, 1968). The results were consistent with the proposal (Cooper & Helmstetter, 1968) that the time taken to replicate the chromosome, C in their terminology, is constant at 41 minutes over the range of conditions (20 min$\leq\tau\leq$60 min) used in their experiments. Their data were entirely compatible with the existence of dichotomous replication in rapidly growing cells and a gap between rounds of replication in slowly growing cultures. At very slow growth rates the situation regarding the velocity of replication and length of the replication period is less clear and a point of controversy has arisen. On the one hand experiments involving the use of synchronous cultures have led to the conclusion that the replication period lengthens in proportion to the generation time (Gudas & Pardee, 1974; Helmstetter, 1974; Helmstetter, et al. 1968), whereas measurements taken on exponential steady-state cultures are consistent with a constant replication period even at slow growth rates. For instance analyses of the

cellular DNA content (Kubitschek & Freedman, 1971) and of the gradient of chromosomal marker frequencies (Chandler, Bird & Caro, 1975), as functions of growth rate both support this view. Which of these two possibilities is the true situation remains to be resolved.

In addition to demonstrating the constancy of the replication period C, the data of Cooper and Helmstetter also show that the interval, D, between completion of a round of replication and the following cell division is also independent of growth rate. Thus division always occurs a fixed time (C+D), after initiation. By combining this knowledge with the data of Schaechter et al. (1958) on the relationship between the average size of S.typhimurium cells at various growth rates, Donachie (1968) was able to demonstrate that the cell size at initiation is "remarkably constant". This relationship between cell size and the number of chromosome origins present at initiation can constitute the basis of a self regulating biological clock to control the frequency of initiation (Pritchard, 1969). Various models have been formulated to suggest ways in which this control may be exerted (Helmstetter et al. 1968; Marvin, 1968; Pritchard et al. 1969; Sompayrac & Maaløe, 1973). To date there is no clear evidence on the molecular nature of the initiation process, although data have been presented to show that RNA (Lark, 1972) and specific protein synthesis (Lark & Renger, 1969; Ward & Glaser, 1969) may be involved.

The suggestion that the rate of replication at a growing point could be altered by external factors,

independently of the growth rate, was first raised by Pritchard and Zaritsky (1970). Their idea arose from an earlier observation which had been largely overlooked. Friesen and Maaløe (1965) had reported that the rate of DNA synthesis in cultures of thymineless mutants of E.coli could be transiently altered by transferring the cells to a different thymine concentration. They were able to exclude leakiness of the Thy⁻ mutation as the cause of this behaviour, but made no further comment on the significance of their observation. Subsequently it was found (Beacham, Beacham, Zaritsky & Pritchard, 1971) that the size of the intracellular thymidine triphosphate pool in such strains is influenced by the concentration of exogenous thymine supplied to the culture. Pritchard and Zaritsky argued that reduction in thymine concentration may impose a substrate limitation for thymidylate on the replication process and therefore lead to a reduction in the rate of DNA synthesis at a replication fork. They were able to substantiate their arguments by demonstrating (Pritchard & Zaritsky, 1970; Zaritsky & Pritchard, 1971), that in cultures of two different thymine requiring strains, E.coli K12, CR34 and E.coli 15T⁻ 555-7, the DNA/mass ratio of the culture was dependent upon the thymine concentration in the growth medium. Since the DNA/mass ratio ($\bar{G}/\bar{M}$) is a function of the replication period C and the doubling time τ (see equation 4), which is unaffected by thymine limitation, the changes observed in $\bar{G}/\bar{M}$ are attributed to a lengthening of the C period in low thymine. The magnitudes of the replication times which can be calculated in this way are

only relative, since a reference C value has to be assumed. However, the calculated size of the change (ΔC) is unaffected by the value of C chosen for the reference point. The effect of thymine limitation on replication velocity was confirmed by another series of experiments in which the increments in DNA (ΔG) synthesised during amino acid starvation in various thymine concentrations were measured (Pritchard & Zaritsky, 1970). ΔG can also be expressed as a function of C and τ (see equation 5) and therefore provides an alternative method for measuring C changes. The values of C estimated in this way are absolute since no reference point is necessary for the calculations. Furthermore, ΔG is solely dependent upon C and τ and independent of the initiation mass (k), in contrast to $\bar{G}/\bar{M}$. Therefore changes in ΔG as a result of growth in different thymine concentrations can be attributed to changes in replication velocity only. It could be argued that changes in $\bar{G}/\bar{M}$ arise by variation of the initiation mass with thymine concentration. The ΔG measurements do have one potential source of error in that it is assumed that all rounds of replication in progress at the time of inhibition of protein synthesis continue to completion (Maaløe & Hanawalt, 1961). This assumption has recently been questioned (Marunouchi & Messer, 1973).

A consequence of the decrease in DNA/mass ratio brought about by thymine limitation is a reduction in the dosage of all genes, other than those located at the replication origin, (Chandler, 1973; Chandler & Pritchard, 1975; Pritchard, 1974). By using gene output as a

measure of gene concentration, Chandler has shown (Chandler, 1973; Chandler & Pritchard, 1975) that the size of the change in concentration of any gene, for a given change in DNA/mass ratio, is dependent upon the relationship between the gene and the origin of replication. Genes located at the terminus experience a greater reduction in concentration than those situated nearer the origin. This is also evidenced by the enhanced gradient in chromosomal marker frequencies during growth in low thymine (Bird et al. 1972). That the magnitude of the alteration in gene concentration is dependent upon the position of the gene in the replication sequence confirms the view that it is the rate of progression of forks which is affected by thymine limitation rather than the mass at which initiation of replication takes place. More directly Manor, Deutscher and Littauer (1971) have found that during growth in low thymine the step-time for the addition of deoxyribonucleotides to the growing DNA chain is extended.

The discovery that the replication velocity can be altered without concomitant change in growth rate provides a corollary to the observations of Cooper and Helmstetter (1968) and a convincing demonstration of the insight in the original hypothesis (Maaløe, 1961) of a disjunction between the rate of DNA chain elongation and cell growth.

The ability to vary the replication time has been used in this study to investigate the temporal relationship between chromosome replication and cell division.

1.III. BACTERIAL GROWTH AND DIVISION

During steady-state growth the rod-shaped bacteria increase in size by extension in length only (Adler & Hardigree, 1964; Donachie & Begg, 1970; Marr, Harvey & Trentini, 1966; Schaechter *et al.* 1962) and eventually divide to produce two equivalent progeny cells.

Measurement of the radioactivity incorporated into *E.coli* cells of different ages and sizes during brief exposure to a labelled precursor of RNA or protein, indicates that the synthesis of these macromolecules increases during the cell cycle (Dennis, 1971; Ecker & Kokaisl, 1969; Manor & Haselkorn, 1967). The proportionality between cell size and rate of synthesis leads to the conclusion that the increase in macromolecular mass is exponential with age (Ecker & Kokaisl, 1969). In contrast, studies on synchronous populations suggest that cell volume increase proceeds in a linear manner (Kubitschek, 1968a,b; Ward & Glaser, 1971). Combination of this observation with that of the constancy of cell width during growth (Marr *et al.* 1966) is suggestive of a linear mode of surface enlargement. An interesting possible consequence of exponential mass increase and linearly increasing volume, and surface, is that the cell may experience increasing hydrostatic pressure which could cause the extension in length (Previc, 1970). Only by increasing the rate of surface growth relative to mass could the cell relieve this internal pressure and so achieve suitable conditions for division (Previc, 1970). Clark (1968a) has examined

the resistance of E.coli of different ages to sonication, a treatment which kills by causing rupture of the cell wall. He observes an oscillation in the size of the surviving fraction over the cell cycle. Maximal resistance is found at the time of division while the minimum occurs some 15 minutes before hand. This may be a reflection of an oscillating cell turgor, although he attributes it to compartmentalisation of the cell before physiological division.

A constant rate of growth with age has been demonstrated for the synthesis of the rigid layer of the E.coli B/r cell wall, murein (Hoffmann, Messer & Schwarz, 1972). There is a discrete doubling in rate about 20 minutes before division,which is also the time of maximal sensitivity of the cell to the cell wall synthesis inhibitor penicillin. This probably denotes the time of maximum autolytic enzyme activity (R. Hakenbeck & W. Messer, unpublished data). Discontinuous linear synthesis has also been reported for the membrane proteins of another rod-shaped organism, B.subtilis (Sargent, 1973). In this case the situation is complicated by an efflux of proteins from the membrane prior to division. Preliminary studies with synchronous cultures of E.coli B/r indicate that here too the rate of synthesis of certain envelope proteins may take place in a stepwise manner (G.G. Churchward, personal communication; R. Hakenbeck & W. Messer, unpublished results).

A linear rate of synthesis is not incompatible with a zonal mode of surface growth, and its implications for

chromosome segregation, as previously suggested (Clark, 1968b; Jacob, Brenner and Cuzin, 1963; Sargent, 1974). The increased rate of synthesis may result from the introduction of new growth sites. Only in the case of the spherical bacterium Streptococcus faecalis has the mode of surface growth been clearly defined. Morphological investigations (Higgins, Pooley & Shockman, 1971; Higgins & Shockman, 1970) show that new wall is primarily incorporated as a double layer at the site of the incipient cross wall. This double wall is peeled apart by autolytic enzyme action and growth of the cell to give the characteristic spherical appearance, and new growth sites are introduced at the junctions of old and new wall once per generation (Higgins & Shockman, 1971).

In the rod-shaped species the situation is less clear. Studies using surface markers such as permease molecules (Autissier & Kepes, 1971; Autissier, Jaffe & Kepes, 1971) and phage receptor sites (Begg & Donachie, 1973; Leal & Marcovich, 1971) indicate conservation of large areas of preformed envelope and therefore localised insertion of new material. The segregation of flagellae during growth of a mutant of B.subtilis which is temperature sensitive for their synthesis shows a distribution which is consistent with a small number of localised regions of surface enlargement (Ryter, 1971). Pulse-labelling experiments (Ryter, Hirota & Schwarz, 1973) have shown that integration of diaminopimelic acid into the murein sacculus (Weidel & Pelzer, 1964) occurs primarily at a central zone in cells of all ages in both fast and slow growing cultures. This

newly made material is rapidly dispersed over the entire surface thus accounting for the previously observed patterns of segregation (Linn et al. 1971; Tubergen & Setlow, 1961) which had been interpreted as evidence for a random intercalatory mode of wall synthesis. The localisation of the hernia induced by growth in the presence of penicillin, and suggested to indicate the site of sacculus enlargement (Schwarz, Asmus & Frank, 1969), has been found to vary with cell age and growth conditions (Donachie & Begg, 1970). The constancy of the distance between this site and one cell pole in slowly growing cells has been presented as evidence for a polarised mode of surface growth from discrete sites. The authors propose that new growth sites, each capable of synthesising a fixed amount of cell envelope, are introduced with each act of initiation of a round of chromosome replication so that the cell can be considered as several discrete "units". Thus the size of the cell is dependent upon the number of chromosome initiations which have taken place.

Division of E.coli normally occurs midway between the cell poles by invagination of a double layer of envelope (Burdett & Murray, 1974) which is subsequently parted as the two cells separate. It has been suggested by Clark (1968b) that cells become compartmentalised prior to physical separation. By examining the division pattern of a strain which is mutant in the placement of its division sites Teather, Collins and Donachie (1974) have shown that the number of events which can occur is strictly regulated by the amount of cell growth.

Two further sets of observations reflect upon the nature of cell growth. Schaechter et al. (1958) report that the increase in cell mass resulting from a nutritional stimulation of growth rate is accommodated by an extension not only in cell length but also in width. Thus although a fast growing cell may have double the mass of a slowly growing cell, and each grows only in length, it does not follow that the larger cell will have twice the length of its smaller counterpart. Similarly the increase in size due to a lengthening of the replication period independently of growth rate, is achieved largely by an increase in cell width (Zaritsky & Pritchard, 1973). In both cases, "shift-up" and "step-down", in moving from one growth state to another cells experience a change in shape which results in a reduction in their surface area/volume ratio; i.e. they become more spherical. It is suggested (Pritchard, 1974; Zaritsky & Pritchard, 1973) that these morphological changes may reflect the action of a regulatory mechanism, determining the rate of envelope synthesis.

1.IV. THE CONTROL OF CELL DIVISION

The thymine starvation experiments of Barner and Cohen (1956) provided some of the earliest evidence of a connection between DNA replication and cell division. The occurrence of a burst of rapid division some 45 minutes after the re-addition of thymine to a starved culture has been attributed to the attainment of a critical DNA/mass ratio (Donachie, Hobbs & Masters, 1968). Walker and

Pardee (1968) have shown that irradiation of cells with ultraviolet light, a known inhibitor of DNA synthesis, can result in an inhibition of cell division. The increased sensitivity to the radiation after incorporation of bromodeoxyuridine led them to conclude that a relationship must exist between DNA metabolism and septum formation.

A coupling between a specific stage of the replication cycle and cell division was first suggested by Clark and Maaløe (1967). They observed a "rather constant time, about 20 to 30 minutes" between initiation and division at three growth rates and concluded that "cell division is triggered at the time of initiation or soon after, say when a particular cistron is replicated". The precise temporal relationship was elucidated by Helmstetter and Cooper (1968). In addition to their suggestion of a constant replication period C, they proposed (Cooper & Helmstetter, 1968) the existence of a second constant period D, between termination of a round of replication and division. Thus each initiation event is followed C+D minutes later by cell division.

For membrane-bound populations of E.coli B/r C and D remain constant at 41 and 22 minutes respectively. However, as for the C period, the length of the D period under some growth conditions is a matter of dispute. Synchronous culture and membrane elution data of both E.coli and S.typhimurium are interpreted to show a lengthening D which occupies a constant fraction of the generation time as the growth rate is reduced, (Cooper & Ruettinger, 1973; Helmstetter, 1974; Helmstetter et al. 1968). In contrast measurements on exponential steady-

state cultures indicate an invariant value of about 25 minutes for D even in slowly growing cultures (Kubitschek, 1974; Kubitschek & Freedman, 1971).

By analysing the age at which division of synchronous cultures becomes immune to an inhibition of DNA synthesis Marunouchi and Messer (1972) have concluded that the D period remains constant at 20 minutes even at low temperatures where the generation time is lengthened. However Pierucci (1972) has produced membrane elution data to show that in each of a variety of media D constitutes a proportion of the generation time which is independent of temperature. This latter finding is in agreement with the observations of Schaechter et al. (1958) on the effect of growth temperature on the composition of steady-state cultures. It is possible that the discrepant result of Marunouchi and Messer may be a consequence of the large variance in the cell age at which cell cycle events take place.

That there is a temporal control on division exerted by the replication cycle has been clearly demonstrated in the "velocity-jump" experiments of Zaritsky and Pritchard (1973). When an exponential culture is "stepped-up" from a slow to fast replication velocity there is a transient acceleration in the rate of division. Similarly in the "step-down" experiment the rate of cell division is observed to be temporarily reduced. Since an alteration in replication velocity transiently affects the frequency at which rounds of chromosome replication are completed the experiments provide good evidence that the time of

division is timed from a late stage of the replication cycle.

The constancy of the D period at fast growth rates has been the subject of much conjecture and various suggestions regarding its significance in the bacterial cell cycle have been forwarded. Investigations into the effects of an inhibition of DNA synthesis on cell division in both synchronous and exponential cultures have led Clark (1968a,b) and Helmstetter and Pierucci (1968) to propose that termination of a round of replication provides a signal for cell division to follow D minutes later. Evidence in support of this view has been forwarded by Hoffmann et al. (1972) from their finding that the rate of murein synthesis increases at a late stage of the division cycle. These authors postulate that termination is a signal for polar cap formation and D therefore represents the time necessary for synthesis and assembly of the crosswall.

On the other hand it has been argued that since division can continue in the absence of DNA synthesis when temperature sensitive initiation defective mutants of E.coli and S.typhimurium are shifted to the non-permissive temperature (Hirota, Jacob, Ryter, Buttin & Nakai, 1968; Spratt & Rowbury, 1971), processes leading to division might be triggered earlier in the cell cycle, perhaps at the time of initiation and proceed independently of on-going replication (Jones & Donachie, 1973; Shannon & Rowbury, 1972). Although it is also assumed by these authors that termination of a round of replication is a

requirement for division it is argued that it does not determine the time at which it occurs : D merely represents the time difference between completion of these "division-processes" (C+D), and termination of the round of replication which was initiated at the same time.

An alternative model for the temporal regulation of cell division has been formulated by Zaritsky and Pritchard (Pritchard, 1974; Zaritsky & Pritchard, 1973; see also Bazill, 1967; Higgins & Shockman, 1971; Previc, 1970). It is proposed, on the basis of data about cell growth, that cell envelope synthesis takes place at a linear rate which is coupled to the replication cycle such that completion of each round of replication results in a doubling in rate. This could arise either by an equivalence between the number of growth sites and chromosome termini or by constitutive production of the rate-limiting envelope component from a gene located near the chromosome terminus. Division occurs as a consequence of the linearly growing envelope, and hence volume, and the exponentially increasing mass. As the cell is making mass faster than envelope during the early part of its cycle there is an increasing pressure within the cell causing extension in length. At termination the rate of envelope synthesis exceeds that of mass, the pressure is relieved and the surplus envelope not required for volume increase is utilised for septum formation.

The requirement of protein synthesis for cell division is less clear. Various workers (Reeves & Clark, 1972a,b; Reeves, Groves & Clark, 1970; Smith & Pardee, 1970) have presented evidence in support of claims for a gradual

accumulation, with growth, of a specific thermolabile protein which is consumed in the division process. Studies on the division pattern of synchronous cultures following readdition of a required amino acid, have led to the suggestion (Pierucci & Helmstetter, 1969) that a period of protein synthesis, which normally runs concurrent with the replication process is required in order for cells to enter the D period. Once this condition has been fulfilled cells are able to proceed to division independently of on-going protein and DNA synthesis. The use of inhibitors of protein and RNA synthesis reveals that production of a specific protein, "termination protein", at the time of termination of chromosomal replication is needed for division (Jones & Donachie, 1973). This view has been extended by Zusman and co-workers (Zusman, Inouye & Pardee, 1972) who suggest that the division triggering mechanism may involve an interaction between this protein and a small metabolite.

Recently some of these views have been questioned. Lomnitzer and Ron (1972) have shown that the synchronisation of cell division after transfer of an exponential culture to a higher temperature, which has been claimed as evidence for a thermolabile division protein (Smith & Pardee, 1970), may be due to an unintentionally imposed limitation in methionine biosynthesis. It is therefore possible that other reported aberrations in cell division are also caused by indirect mechanisms.

Marunouchi and Messer (1973) have shown that protein synthesis at the time of termination is required for

completion of the replication process. This conclusion has also been reached by Dix and Helmstetter (1973).

1.V. OBJECTIVES OF THE RESEARCH PROJECT

The observed sequence of events of the bacterial division cycle has been attributed to various relationships between the major macromolecular biosynthetic processes. Since none of the models so far suggested to explain the coupling between replication and division have been conclusively proven or disproven, the situation warrants further investigation.

A method by which the two "temporal-sequence" models might be distinguished has been outlined by Zaritsky and Pritchard (1973). It exploits their discovery that the replication velocity can be varied independently of the growth rate. If as these authors have demonstrated, this results in an extension of the replication period C, then an analysis of any accompanying changes in the D period will differentiate between the two models. For although both explain the constant time interval (C+D) between initiation and division, they lead to different predictions about the length of D under conditions of changing C. If termination sets into operation a sequence of events resulting in cell division then D should remain constant and independent of changes in C, whereas if initiation provides a signal an inverse relationship between C and D is predicted so that as C is increased D will decrease.

In this study two methods are used to examine the effects of changing replication velocity upon the length of the D period. The most direct analysis is obtained from an examination of the pattern of chromosome replication during the cell cycle by means of an age-fractionation technique. This method, membrane elution (Helmstetter, 1967), is that by which C and D were originally defined (Cooper & Helmstetter, 1968). Changes in the length of D will be detected as changes in the cell age of termination. If there is an increase in C equal to a decrease in D there should be no variation in the time of initiation but termination will appear later in the cell cycle. If on the other hand, D is independent of changes in C, there will be no effect on termination but initiation will move progressively earlier in the division cycle. Thus this analysis should detect not only changes in D but also the increments in C with which they are associated. A second estimate of D can be obtained from measurements of cell size and DNA content of exponential cultures growing with different replication velocities. Just as changes in the DNA/mass ratio can be used to quantitate changes in C (Pritchard & Zaritsky, 1970) alterations in average cell size can be used to measure changes in (C+D). Hence, by a combination of these two sets of data estimates of D can be obtained for a series of different C times.

The kinetics of transition as a culture moves from one replication velocity to another will reflect upon the nature of the temporal relationship. The extent of any

delay between the transition and the time at which the rate of cell division responds to the altered replication velocity provides a measure of the interval between the triggering-event and the division per se. If division is timed from termination then the rate of cell division will respond to the transition, which transiently alters the frequency of termination, after a delay of D minutes. If division is timed earlier in the replication cycle then the delay will be proportionally longer. Similarly measurement of the fraction of an exponential population able to continue division in the presence of an inhibitor of DNA replication will also provide a measure of the time elapsing between the signal for division and the event itself. If termination triggers division then the extent of division should be equivalent to the proportion of the cell population in the D period.

The alternative to these temporal sequence models, recently offered by Pritchard (1974) proposed that division occurs when a linearly growing envelope, subject to rate regulation by the replication cycle, exceeds exponential mass increase. This model can be tested by examination of the rate of cell envelope synthesis as a function of cell age. If the model is correct then envelope synthesis should show a step pattern, with the doubling in rate associated with a particular stage of the replication cycle. Thus treatments which affect the temporal process of replication, e.g. an inhibition or reduction in rate, should also influence the pattern of envelope synthesis.

Finally if envelope synthesis is subject to this "gene dosage" control then the average envelope/mass ratio of an exponential steady-state culture will be proportional to the concentration of the gene responsible for exerting the control. Changes in the gene concentration effected by changes in replication velocity (Chandler, 1973; Chandler & Pritchard, 1975) should result in predictable variations in the envelope/mass ratio of the culture.

2. MATERIALS AND METHODS

2.I. BACTERIA

The strain used for this investigation, LEB16, is an F^- *lacZ*, *str*, segregant of an F'*lac*$^+$ *E.coli B/r* obtained from Dr. M. Pato. It carries chromosomal mutations at *thyA* and *drm*, thereby enabling it to grow in the presence of low concentrations of thymine i.e. less than 20 µg/ml. LEB18 is a spontaneous *Thy*$^+$ derivative of LEB16.

P178 is the *E.coli* $15T^-$ (555-7) which has previously been used by Pritchard and Zaritsky (1970). It requires additional supplements of arginine, methionine and tryptophan for growth.

2.II MEDIA

The minimal salts medium used was that described by Helmstetter (1967) which has the composition: NH_4Cl 2g; Na_2HPO_4 6g; KH_2PO_4 3g; NaCl 3g; $MgSO_4$ 0.25g; H_2O 1l. Particulate matter was removed from the media by filtration through a membrane filter (Sartorius 0.45 µm pore size). Glucose (0.2% w/v), glycerol (0.2% w/v) or a mixture of L-Proline and L-Alanine (0.4% w/v) were added as carbon sources. These routinely gave generation times of 42 ± 2; 55 ± 2 and 65 ± 3 minutes respectively. Additional L-amino acids were added (50 µg/ml) as required.

Thymine, the only variable, was added at concentrations as indicated in the text.

Stock cultures of bacteria were maintained on M9 minimal agar containing appropriate supplements.

2.III. RADIOACTIVE CHEMICALS

Radioactively labelled chemicals were used in these studies to investigate both the rate of synthesis, and cellular content of various macromolecules.

L - [U-14C] Leucine (270 mCi/mmole), 2-14C Thymidine (50 mCi/mmole), [methyl-3H] - Thymidine (19 Ci/mmole), 2-14C Thymine (50 mCi/mmole) and 2-14C Uracil (50 mCi/mmole) were obtained from The Radiochemical Centre, Amersham. D- [1-14C] Glutamic acid (10-30 mCi/mmole) was obtained from Calatomic, Los Angeles.

2.IV. MEASUREMENTS ON STEADY-STATE EXPONENTIAL CULTURES

Growth medium was inoculated with bacteria from a minimal agar plate and incubated with vigorous aeration at 37°C in a New Brunswick gyratory shaker.

Samples were taken over several generations of growth and only when the culture was in a steady-state of exponential growth (Campbell, 1957; Pritchard & Zaritsky, 1970).

Changes in thymine concentration during the course of an experiment were achieved either by dilution to the culture with prewarmed medium containing thymine sufficient to give the required final concentration, or by filtration

of the culture onto a membrane filter (Millipore 0.45 μm pore size) and resuspension in prewarmed medium containing thymine as required.

Culture mass was measured as absorbance at 450 nm (A_{450}) by means of a Gilford microsample spectrophotometer with a 1 cm path length. Previous investigators (Collins, 1971; Schaechter et al. 1958) have shown that this provides an accurate measurement of cell mass when the cells are obtained from steady-state exponential cultures. An A_{450} of 0.10 is equivalent to about 16 μg/ml dry weight. For measurements of steady-state composition the absorbance was kept in the range 0.05 to 0.2 by dilution with fresh prewarmed growth medium after each doubling in absorbance.

Particle number was determined by the use of the Coulter Counter (Model B; 30 μm orifice) on samples taken into ice cold saline (0.9% w/v) with formaldehyde (0.4% w/v final concentration).

Relative DNA contents were measured by the incorporation of 2-14C thymine (0.05 μCi/μg) from the growth medium into trichloracetic acid (TCA) precipitable material. Samples were taken into equal volumes of 10% TCA and held at 0°C for at least 30 minutes. The samples were then collected by suction onto 27 mm membrane filters (Sartorius, 0.45 μm pore size) which were washed six times with 20 ml 96°C distilled water and dried under an infra-red lamp. The filters were placed with constant orientation in small vials which were filled with scintillation fluid (PPO 5g. POPOP 0.3g Toluene 11, Pritchard & Lark, 1964), stoppered and placed in turn into Standard Packard vials. The

samples were counted in a Packard liquid scintillation counter.

2.V. MEMBRANE ELUTION TECHNIQUE

The membrane elution technique described by Helmstetter and Cummings (1964) and developed later by Helmstetter (1967) depends upon the ability of E.coli B/r to bind to, and grow on, membrane filters in the presence of growth medium. If an exponentially growing culture is bound to such a filter, the filter inverted and growth medium pumped through the filter then the cells will grow and divide, and on average one of each pair of daughter cells will be eluted from the membrane. Therefore by continuous sampling of the eluate the progeny can be fractionated according to the ages of their parents at the time of binding to the membrane, because the first cells to be eluted will be those resulting from divisions of the oldest cells. Only at progressively later times will cells be produced as a result of divisions of cells that were at progressively earlier cell ages when bound. If the culture is pulsed with a radioactively labelled macromolecule precursor prior to binding, then measurement of the amount of label contained in the eluted progeny will give an estimate of the variations in the rate of synthesis of the macromolecule over the cell cycle. This of course, assumes equal partition of the label incorporated into the parent between the two daughter cells.

a) Apparatus

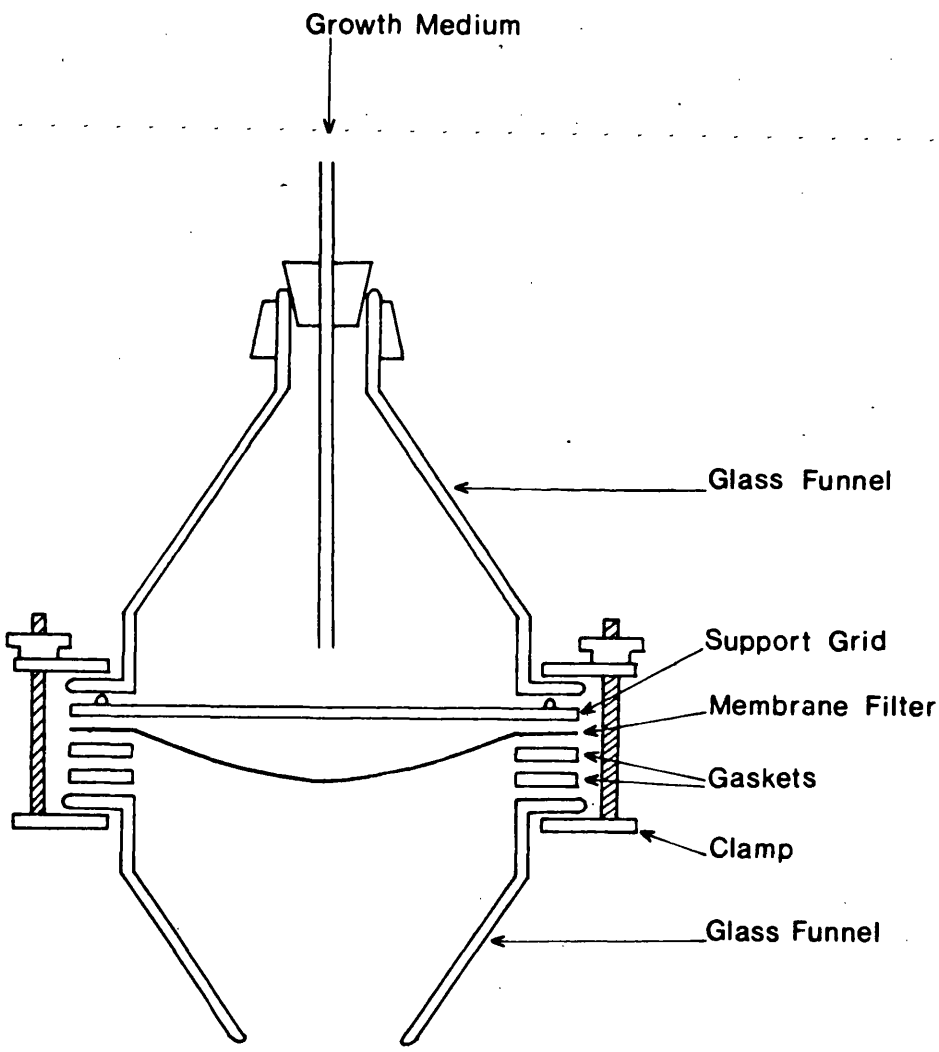
The apparatus used in this study and shown diagrammatically in Fig. 1 is similar to that described by Helmstetter (1969). It consists of two funnels each constructed by joining a 100 mm Pyrex glass filter funnel to the ground glass flange of a Quickfit reaction vessel, which are clamped either side of a 142 mm diameter Stainless Steel support grid, (Millipore Cat No. YY2214264). Watertight seals between the steel grid and the two glass flanges were achieved by use of a Teflon 'O'-ring and two gaskets cut from $\frac{1}{8}$ inch IRCOL rubber. One funnel was fitted with a 25 mm diameter end-tube to facilitate connections to a suction pump for filtering cultures onto the membrane, and to the medium supply during elution. The other funnel was truncated so that the eluate could drop freely from the filter. During use a 142 mm grade GS Millipore membrane (0.22 μm pore size), which had previously been soaked for at least 30 minutes in warm elution medium, was fitted into the apparatus. Prewarmed elution medium was pumped to the elution apparatus by means of a peristaltic pump, (Buckler Instruments Polystatic Pump).

b) Experimental Procedure

100 ml exponential cultures at a cell density of approximately 10^8/ml, and containing thymine at the required concentration, were pulsed for 3 to 4 minutes with a radioactively labelled precursor for the macro-

Figure 1 : MEMBRANE ELUTION APPARATUS

Diagram to show the construction of the elution apparatus. The figure is not drawn to scale.


Growth Medium
Glass Funnel
Support Grid
Membrane Filter
Gaskets
Clamp
Glass Funnel

molecule under investigation. The pulse was ended by binding the cells to the surface of the Millipore membrane. The cells were washed with 100 ml minimal medium and the apparatus inverted for connection to the medium supply and elution of the dividing cells. Care was taken during this binding and pulse terminating process, which usually took less than 2 minutes, to ensure that at no time did the filter go dry, which would have exposed the cells to a transient starvation period. Elution rate was rapid (22 ml/min) for the first 6 minutes in order to remove the majority of "unbound" cells and then reduced (6 ml/min) for the remainder of the elution period. Samples of the eluate were collected continuously throughout this period. To ensure that the apparatus at all times remained at a constant temperature the experiments were carried out in a 37°C constant temperature room.

Cell density in the eluate was determined with an electronic particle counter (Coulter Model B) fitted with a 30 μm orifice, on samples diluted into isotonic saline. Radioactivity in the eluted cells was measured by the amount of label contained in the 5% TCA insoluble material of 10 ml samples of the eluate.

It was found that prewarmed fresh medium could be used in place of conditioned medium (Helmstetter and Cummings, 1964) with no apparent difference to the growth of the cells.

c) Analysis and Interpretation of results obtained by this technique

As outlined previously, the membrane elution technique provides a method of fractionating cells according to their ages at the start of the experiment. Since the cells on the membrane divide from the oldest through to the youngest, then the elution profile i.e. the density of eluted progeny cells with time, should reflect the age distribution of the cells in the original exponential population.

The form of this distribution is given by equation (1), (see Introduction).

I have used the equation to produce the theoretical curve for the idealised age distribution in an exponential culture containing no dispersion in the generation times of individual cells, and the elution profile expected from such a population (Fig. 2). Also shown is a typical elution profile obtained from LEB16 growing in the PA medium with 20 µg/ml thymine, (doubling time ca 65 minutes). The shape of the experimental curve is clearly very similar to that of the predicted one. The general roundedness of the profile i.e. the deviation from a sharp saw-tooth pattern, indicates the extent of the dispersion of doubling times in the population.

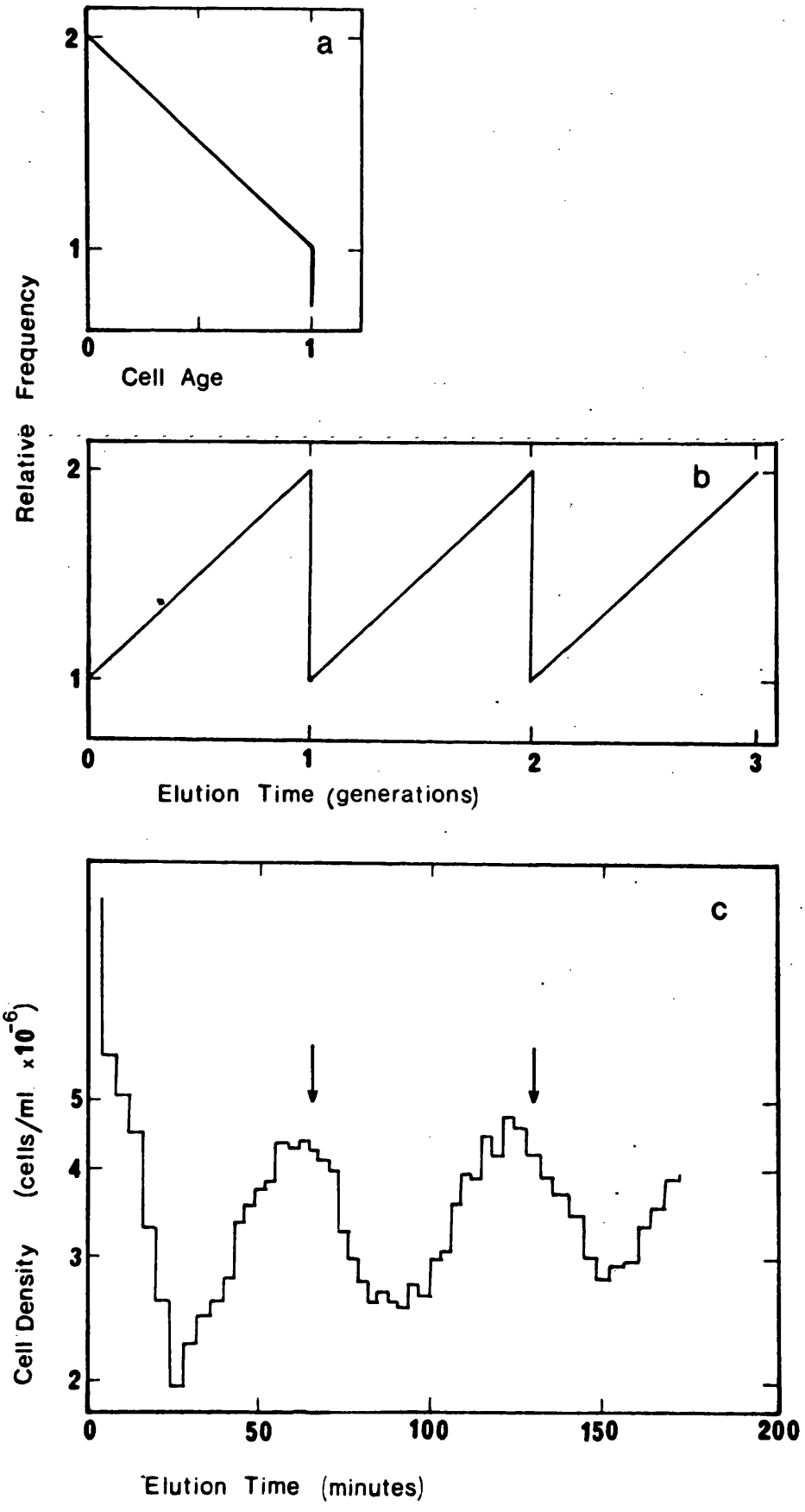
Since the effect of increasing this variance in such a population is to cause a flattening of the elution profile, an estimate of the degree of deviation from ideality can be obtained from the ratio of the height of the maxima to the minima. In the ideal situation i.e. no

Figure 2 : PREDICTED AND OBSERVED ELUTION PATTERNS FOR LEB16

a) Idealised age distribution for an exponential culture growing with a generation time of 65 minutes, calculated from equation (1) (Powell, 1956).

b) Theoretical concentration of effluent cells from a membrane-bound culture with an age distribution as shown in (a).

c) Cell density in consecutive samples of the eluate from a membrane-bound population of LEB16 growing in PA medium containing 20 µg/ml thymine.


a
2
1
0
1
Cell Age
Relative Frequency
b
2
1
0
1
2
3
Elution Time (generations)
c
Cell Density (cells/ml x10^-6)
5
4
3
2
0
50
100
150
200
Elution Time (minutes)

variance, this ratio should be 2 and as the variance increases so this ratio will decrease towards unity. In the curve shown in Fig. 2c the ratio of the first peak height to the second dip is approximately 1.65. I have used the dip following the peak to calculate this ratio because the first dip of the elution profile, at the commencement of the elution, is obscurred by the wash off of "unbound" cells as indicated by the fall in cell density during the first 15 to 20 minutes.

The dispersion of generation times, is an important consideration in membrane elution experiments, especially if a series of measurements are made to examine the trend of changes in cell cycle events with changing environments. It is important to ensure that under these conditions any increase in variance does not contribute significantly to the observed pattern of changes.

An analysis of the amount of radioactivity contained in the eluted progeny as a result of briefly exposing the exponential population to a labelled precursor will allow an estimate of the rate of synthesis of the macromolecule under investigation during the cell cycle (Helmstetter, 1967). Constant levels of radioactivity per cell during the elution indicate a uniform rate of synthesis, and discontinuities, either increases or decreases in the amount of label per cell, indicate corresponding fluctuations in the rate of synthesis during the cell cycle. In the case of DNA synthesis these discontinuities probably reflect initiation and termination of rounds of chromosomal replication.

2.VI. SYNCHRONOUS CULTURES

Since all of the cells collected during elution from a membrane bound population are newborn, a sample should grow synchronously when incubated under suitable conditions.

Cultures of LEB16 were bound to and eluted from the membrane as described previously. After 20 minutes of elution, when the "unbound" cells had been removed from the membrane, the eluate was collected for 80 to 90 minutes into an ice-cold flask. The cells so collected, were then concentrated by suction onto a Millipore membrane filter (0.45μ pore size) and resuspended in one tenth volume of prewarmed growth medium. When incubated the culture showed synchronous growth for at least two generations. Fig. 3a shows a typical result obtained by this method.

From the plot of percentage increment in cell number against time, Fig. 3b it can be seen that the distribution of doubling times is somewhat skewed. This is probably because cells continue to grow, albeit slowly, towards division in the ice-cold collection vessel so that cells harvested at the start of the elution will divide slightly sooner, in the synchronous culture, than those collected at later times.

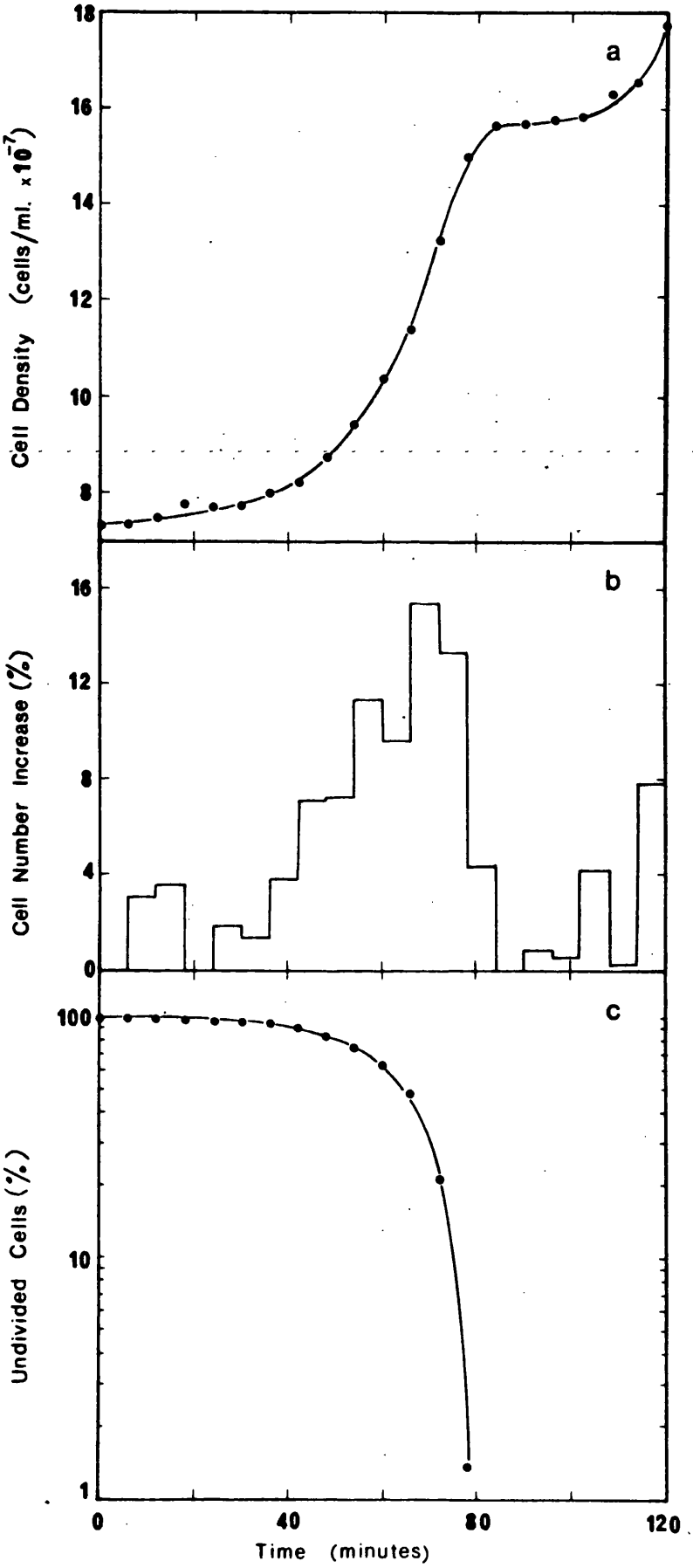
The data is also presented (Fig. 3c) in the form of an "Alpha plot" (Smith & Martin, 1973). This plot of percentage undivided cells against time indicates that the probability of division increases with time. It does not support the hypothesis that the entry of cells into a

Figure 3 : SYNCHRONOUS GROWTH OF LEB16

a) Total cell counts plotted against time after concentration of the eluted cells, collected from the membrane-bound population.

b) The rate of cell division. Data from (a) plotted as percentage increase in total cell number per minute.

c) The "Alpha Plot" of Smith and Martin (1973). Data from (a) plotted as percentage of the initial population remaining undivided per minute, α.


a
b
c
Cell Density (cells/ml. x10⁻⁷)
Cell Number Increase (%)
Undivided Cells (%)
Time (minutes)
18
16
14
12
10
8
16
12
8
4
0
100
10
1
0
40
80
120

deterministic phase occurs with a constant probability (Smith & Martin, 1973).

Kinetic studies on the synthesis of various macromolecules during the division cycle were carried out by pulse-labelling with a suitable radioactive precursor. The pulses were ended by addition of an equal volume of 10% TCA containing cold carrier compound (100 µg/ml) and placing the samples at 0°C. After at least 30 minutes at the low temperature, the acid insoluble material in the samples was collected on membrane filters (Sartorious; 0.45 µm pore size) washed at least ten times with either 96°C distilled water or 5% (w/v) TCA and the filters dried under an infra-red lamp. The samples were counted in the Packard liquid scintillation counter.

2.VII. FRACTIONATION OF BACTERIAL CELLS INTO THEIR BIOCHEMICAL CONSTITUENTS

Two methods of fractionation were used to analyse the site of incorporation i.e. which macromolecule?, of certain radioactive precursors. One method was based upon the procedures of Roberts, Abelson, Cowie, Bolton & Britten (1963) and Park and Hancock (1960); it involves sequential removal of macromolecular components of the cell by various chemical and enzymatic degradations. The second method is that described by Lugtenberg and de Haan (1971) which measures the amount of label sensitive to digestion by specific enzymes.

Method 1. (Park and Hancock, 1960; Roberts et al. 1963)

Labelled exponential cultures, normally 10 ml at circa $5x10^7$-10^8 cells/ml, were mixed with equal volumes of 10% TCA and held at 0°C for 30 minutes. After addition of unlabelled TCA-precipitated carrier cells, ($1-2x10^9$) the total suspension was washed 3 times with 5% TCA to remove unincorporated label by centrifugation at 4000xg and resuspension. The suspension which contained only macromolecular material was then fractionated by the method shown in Table 1. As the fractionation is sequential the entire procedure can be carried out in a single centrifuge tube if care is taken to remove all traces of each supernatant with a swab of tissue paper between each manipulation. Samples (0.3 ml or 0.5 ml) were taken from each supernatant and mixed with 9 volumes of "Aqueous" Scintillation fluid (Triton X100 330 ml; Toluene 660 ml; PPO 5g; POPOP 3mg) for determination of the radioactive content. The samples were thoroughly mixed by shaking and counted in the Packard liquid scintillation counter.

Method 2. (Lugtenberg and de Haan, 1971).

10 ml samples of labelled cells (circa $1-2x10^8$/ml) were cooled to 0°C and disrupted by sonication (4x30 second periods at maximum amplitude with 30 second periods for cooling between bursts, from a M.S.E. Ultrasonicator). The mixture was then heated at 100°C for 5 minutes to denature any autolytic or other degradative enzymes, cooled and split 3 ways.

Table 1. SCHEME FOR FRACTIONATION OF E.COLI

Fraction	Treatment	Major contents of fraction
Cold T.C.A. soluble	Hold 5% T.C.A. suspension at 0°C for 30 min; centrifuge at 4000g for 10 min; decant extract	Low molecular weight material
Aqueous Ethanol-soluble	Suspend residue in 5ml 75% (v/v) ethanol in water; Hold at 48°C for 20 min; centrifuge at 7000g for 10 min; decant extract	Ethanol-soluble lipids
Hot T.C.A. soluble	Suspend residue in 5ml 5% T.C.A. Heat at 96°C for 20 min; cool and centrifuge at 17,000g for 20 min; decant supernatant	Breakdown products of nucleic acids
Trypsin sensitive	Wash and suspend residue in 5ml 0.05M NH_4HCO_3 containing 0.005M NH_4OH; add trypsin (final concentration 50 µg/ml) and incubate at 37°C for 4 hrs; centrifuge at 35,000g for 1 hr; decant extract	Trypsin-degraded material (proteins)

Fraction	Treatment	Major contents of fraction
Lysozyme-sensitive	Wash and suspend residue in 5ml 0.01M Tris/HCl buffer, pH 7.8; add lysozyme (final concentration 50 µg/ml) and incubate at 37°C for 16 hrs; centrifuge at 37,000g for 1 hr; decant extract	Lysozyme-degraded material (murein)
Residue	Suspend pellet in 5ml 5% T.C.A.	Undegraded material

(i) To one portion (4 mls) lysozyme was added at a final concentration of (100 µg/ml).

(ii) To a second portion (4 mls) trypsin was added at a final concentration of 100 (µg/ml).

(iii)To a third portion (2 ml) no additions were made.

The mixtures were incubated at 37°C and samples (0.2 ml or 0.5 ml) removed into 10% TCA (0.5 ml) for estimation of the amount of label remaining in macromolecular constituents. Similar samples were also taken during the pretreatment before the digestions.

2.VIII. ELECTRON MICROSCOPY

Electron micrographs of cells mounted on Collodion films by the "Agar Diffusion" method. (Kellenberger and Arber, 1957) were used to make observations on the effects of growth conditions on cellular morphology.

40 ml cultures at about 10^8 organisms/ml were fixed with 4% formaldehyde. After holding at 0°C for 16 hours they were absorbed onto membrane filters, washed with distilled water (4x20 ml) and resuspended in 1 ml particle free distilled water. Sodium docedyl sulphate was added at a final concentration of 0.04% and a few drops of the suspension spread onto a Collodion film. The films were mounted on copper grids and examined in a AEI 802 electron microscope at a magnification of X6300. Measurements of cell dimensions were made from enlarged photomicrographs. Latex spheres of known diameter (0.308µm) were included in cell samples as calibration standards.

3. CHARACTERISTICS OF THE STRAIN LEB16.

3.I. CHOICE OF GROWTH MEDIUM

The experiments described in this thesis were designed to examine effects of variation in replication velocity on the timing of cell division in a thymineless mutant of E.coli B/r, LEB16. Of particular interest was the period D, between termination of a round of replication and the following cell division. In order to investigate whether changing replication velocity had any effect upon the length of D by the membrane elution technique, a growth rate was required such that the cell age at which termination occurred was well separated from the time of initiation and division. At some growth rates the age at which termination occurs is coincident with that of initiation, (see Cooper & Helmstetter, 1968). In these circumstances termination is not detected by this technique because it is obscured by the initiation event.

Two additional points also had to be considered when deciding upon the growth medium to be used in these studies. It has been reported (Pritchard & Zaritsky, 1970; Zaritsky & Pritchard, 1973; Chandler, 1973) that under certain growth conditions, generally low thymine concentrations and fast growth rates, thymineless strains of E.coli never attain balanced growth. As I also wished to compare measurements of cell size and DNA content under steady-state conditions, and to calculate the magnitude of

changes in C and D at different thymine concentrations it was important to ensure that the medium supported balanced growth over the range of thymine concentrations to be used. It was therefore decided to use a relatively slow growth rate to avoid this complication. On the other hand the growth rate should not be so slow that the doubling time τ was greater than C+D, because of the present uncertainty about the length of C and D under these conditions (see Introduction 1.II and 1.IV). Ideally, a medium was required which would give a doubling time equal to the length of (C+D) i.e. about 64 minutes. From reports in the literature (Clark, 1967; Helmstetter, 1967; Zeuthen & Pato, 1971) it would seem that a minimal salts medium with sodium succinate as carbon source (0.2% w/v) would be a suitable choice. However, repeated measurements on the growth of LEB16 in this medium gave irreproducible growth rates; the doubling time generally being between 80 and 90 minutes.

Investigations with various other carbon sources and supplements showed that a minimal salts medium supplemented with L-Proline and L-Alanine (0.4% w/v) as carbon source routinely gave a doubling time of 65 $\pm$ 3 minutes. This medium, termed P.A. medium, was adopted for the major part of the work in this thesis. Details of the growth of LEB16 in this medium at various concentrations of thymine are reported in later sections.

3.II. THE AMOUNT OF THYMINE REQUIRED FOR GROWTH

An assessment of the amount of thymine needed to support growth of LEB16 on PA medium was made on both solid and liquid media.

Solid medium

A 37°C standing overnight culture of LEB16 in PA medium with 20 µg/ml thymine was diluted in phosphate buffer and duplicate 0.1 ml aliquots spread on PA medium agar containing thymine at various concentrations. After incubation for 36 hours at 37°C the number of colonies and their morphology was recorded. Table 2 shows the results. Clearly the number of viable cells remains essentially unaffected by thymine concentrations as low as 0.5 µg/ml. However the reduced size of the colonies in 1 µg/ml and 0.5 µg/ml thymine suggests that at these low concentrations growth may be affected.

Liquid medium

LEB16 was grown at 37°C in PA medium with a range of thymine concentrations and the growth rate determined by the increase in absorbance at 450 nm. The results (Table 2) show that over the range 20 µg/ml to 1.0 µg/ml thymine the growth rate is unchanged. At lower concentrations (0.5 µg/ml) the rate of increase in absorbance is reduced.

Table 2. GROWTH OF LEB16 ON DIFFERENT THYMINE CONCENTRATIONS

Thymine	Solid Medium [a]		Liquid Medium
µg/ml	Viable Count 10^8 cells/ml	Colony Size (mm)	A_{450} doubling time (min)
20	4.6	3 mm	64 ± 2
5	-	-	65 ± 2
2	4.7	3 mm	64 ± 2
1.5	-	-	65 ± 2
1.0	4.3	1 mm	65 ± 3
0.5	4.0	1 mm	72 ± 4
0.2	0	-	100 ± 5
0	0	-	No growth

(a) 36 hour incubation at 37°C

These results indicate only that growth of LEB16 is "normal" in thymine concentrations as low as 1.0 µg/ml as measured by the rate of increase of absorbance. They do not indicate whether or not growth is balanced over this range of thymine concentrations. An analysis of this is reported later.

3.III. LEAKINESS OF THE THYA MUTATION IN LEB16

As the majority of the experiments reported in this thesis depend upon the ability to vary the replication velocity in LEB16 by adjustment of the thymine concentration in the growth medium, it is important to know the extent of the leakiness of the thyA mutation. If the mutation is leaky then the cell will still be converting some uridylate to thymidylate and therefore not all of the thymine in DNA will be derived from that supplied externally. This endogenous synthesis will lead to a progressive lowering of the specific activity of the thymine in DNA as the concentration of radioactive thymine in the growth medium is reduced. Such an effect will result in an over-estimation of the changes in replication velocity when calculated from measurements of the DNA/mass ratio of cultures in various amounts of thymine.

The leakiness of mutations in the thyA gene can be estimated by comparison of the amount of DNA synthesised in the presence and absence of endogenous thymine, as measured by the incorporation of radioactive uracil into

acid-insoluble material, (Friesen & Maaløe, 1965; Hollom, 1969).

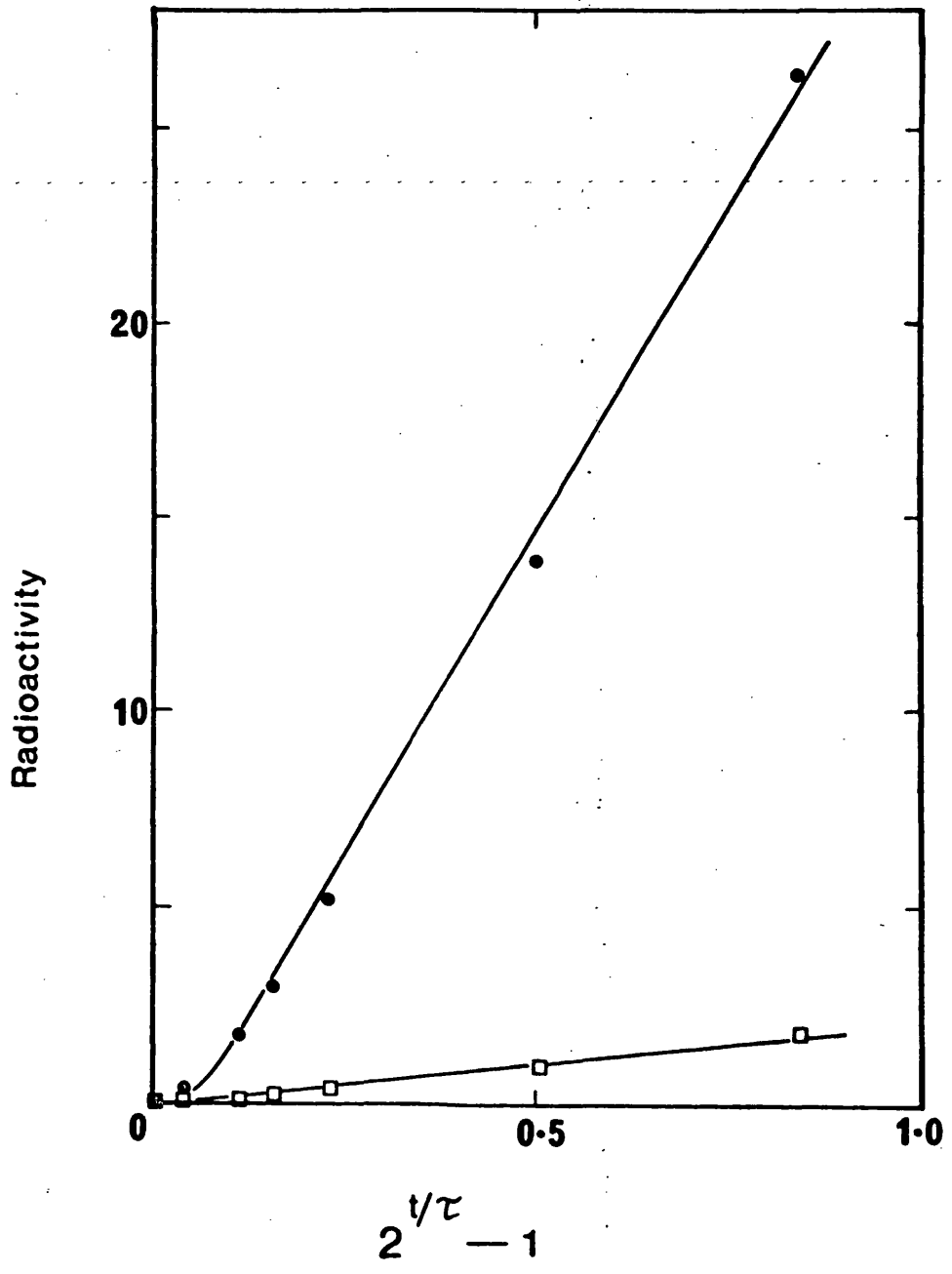
A 37°C 50 ml exponential culture of LEB16, growing in the presence of 4 µg/ml uracil and 4 µg/ml thymine was filtered, washed with medium lacking thymine and then resuspended in 50 ml medium containing ^{14}C uracil (4 µg/ml; 0.1 µCi/µg) but no thymine. The culture was divided into two portions and to one thymine was added at a final concentration of 4 µg/ml. The two cultures were incubated at 37°C and duplicate 1 ml samples withdrawn at time intervals into 1 ml 0.8 M sodium hydroxide (containing 200 µg/ml uracil). The samples were held at 37°C overnight in order to hydrolyse RNA and then neutralised by addition of 1 ml 0.8M hydrochloric acid. After precipitation of the macromolecules with 10% TCA (3 ml), and holding at 0°C for 30 minutes, the amount of radioactive label incorporated into DNA was assayed by collection on membrane filters and use of the liquid scintillation counter.

The data, presented in Fig. 4 in the form of a differential rate plot, show that the rate of incorporation of radioactive uracil into DNA in the absence of thymine is only 7% of that observed when thymine is present, i.e. the rate of DNA synthesis in LEB16 in the absence of thymine is only 7% of that observed in a normal exponential culture. Thus the thyA mutation is no more than 7% leaky.

It could be argued that this method gives an over-estimation of the synthesis in the absence of thymine because under these conditions the radioactive uracil is

Figure 4 : DIFFERENTIAL RATE OF URACIL INCORPORATION

Plots of radioactive uracil incorporated (cpm/ml $x10^{-2}$) into DNA by LEB16 growing in PA medium in the presence (•) and absence (□) of exogenous thymine. The scale on the abscissa is an expanded, logarithmic, scale to allow for growth of the culture during the experiment.


Radioactivity
20
10
0
0·5
1·0
$2^{t/\tau} - 1$

incorporated as both thymidylate and cytidylate. When thymine is present the uracil is incorporated primarily as the latter. Therefore, since E.coli has a G+C content of approximately 50% (see Hayes, 1964), almost twice as many molecules of radioactive uracil will be incorporated per unit of DNA synthesised when thymine is absent. Thus the rate of DNA synthesis in the absence of thymine may be closer to 3.5% of the normal rate. In either case the thyA mutation will not contribute significant amounts of endogenously synthesised thymidylate. For the purposes of this thesis this small contribution will be ignored.

Previous investigators (Friesen & Maaløe, 1965; Hollom, 1969) have provided evidence to show that the estimation of leakiness by this method is comparable to that from more rigorous techniques involving lengthy chromatographic procedures (Cohen & Barner, 1954; Beacham et al. 1971).

3.IV. EFFECT OF CHANGING THYMINE CONCENTRATION ON THE RATE OF DNA SYNTHESIS

The observation that changes in the amount of thymine supplied to thymineless mutants of E.coli can transiently effect the rate of DNA synthesis was recorded first by Friesen and Maaløe (1965) and later by Pritchard and Zaritsky (1970). These latter authors have suggested that this is due to an effect upon the velocity of replication rather than an alteration in the frequency of initiation of rounds of replication. At low concentrations of thymine

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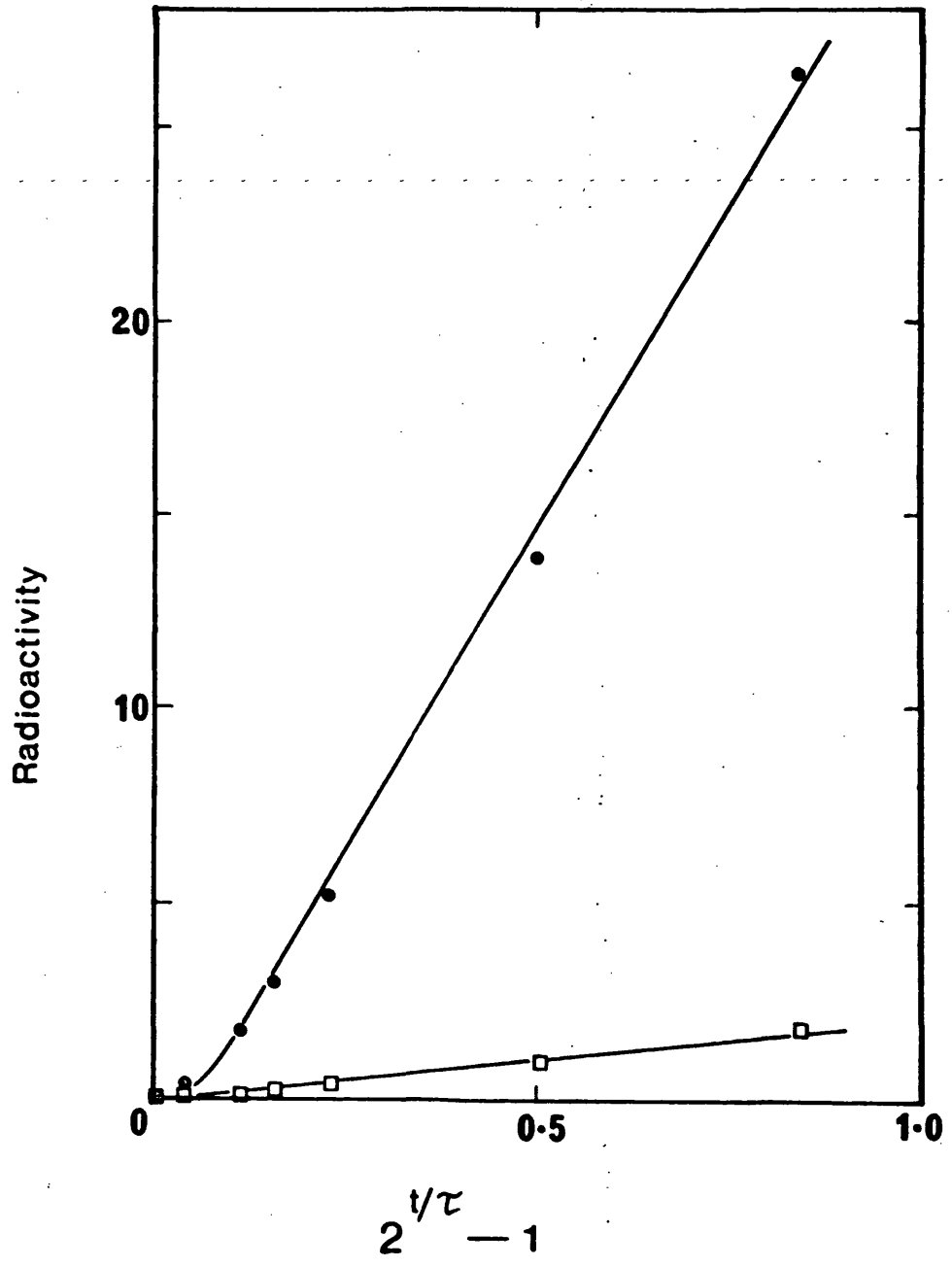
Radioactivity
20
10
0
0·5
1·0
$2^{t/\tau} - 1$

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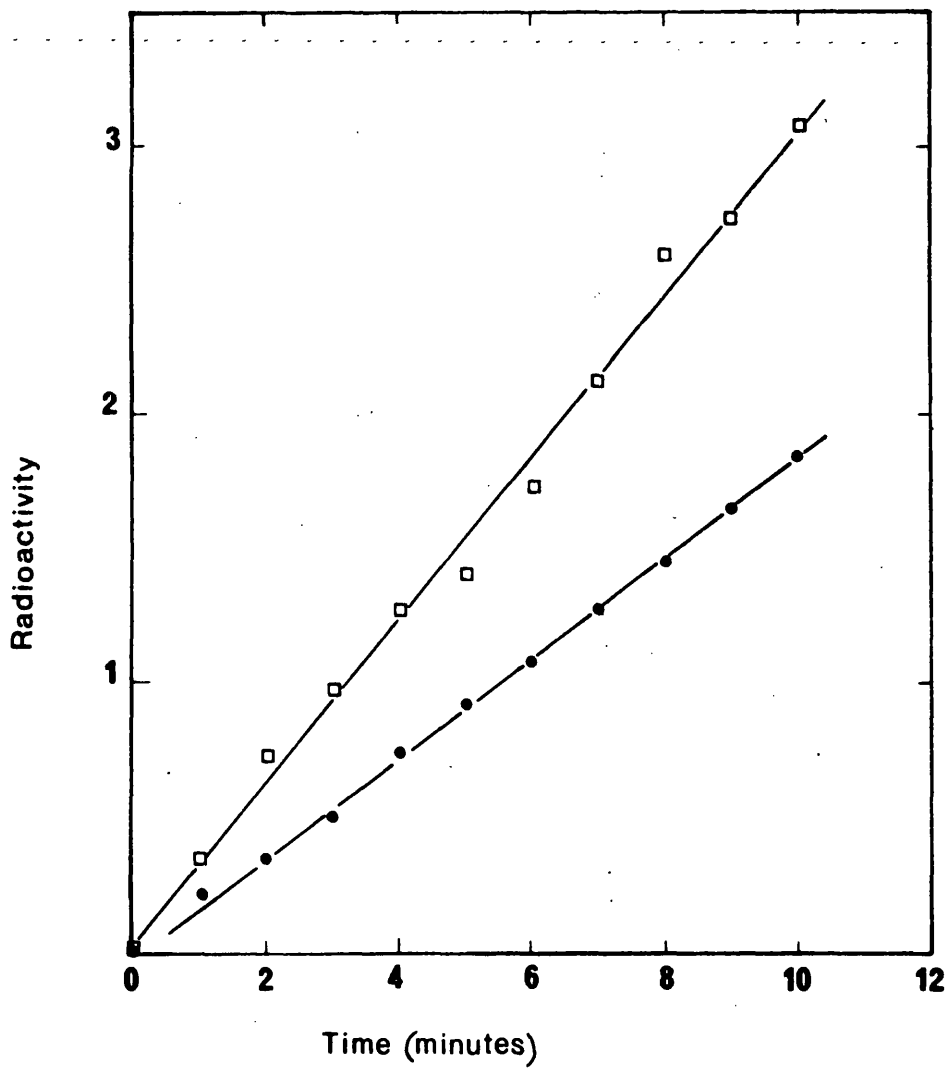
Figure 5 : DNA SYNTHESIS AFTER A "STEP-UP"

Incorporation of radioactive thymine (cpm/mlx10^{-3}) by a culture of LEB16 pregrown in PA medium containing 2 µg/ml unlabelled thymine and transferred, at 0 min, to medium containing ^{14}C-thymine (0.2 µCi/µg) at either (□) 20 µg/ml or (•) 2 µg/ml.


Radioactivity
3
2
1
0
2
4
6
8
10
12
Time (minutes)

the intracellular thymidylate pool is reduced (Beacham *et al.* 1971) which imposes a substrate limitation on the enzymes of the replication process. Thus the velocity of the replication process becomes dependent upon the concentration of substrate.

Fig. 5 shows the result of an experiment in which a culture growing in low (2 µg/ml) thymine was transferred to a higher concentration (20 µg/ml). There is an immediate stimulation in the rate of DNA synthesis of approximately 1.7 fold. The response of LEB16 to changes in thymine concentration is similar to that described for other thymineless mutants of *E.coli* (Pritchard & Zaritsky, 1970).

4. VARIATION OF THYMINE CONCENTRATION AND THE CELL CYCLE

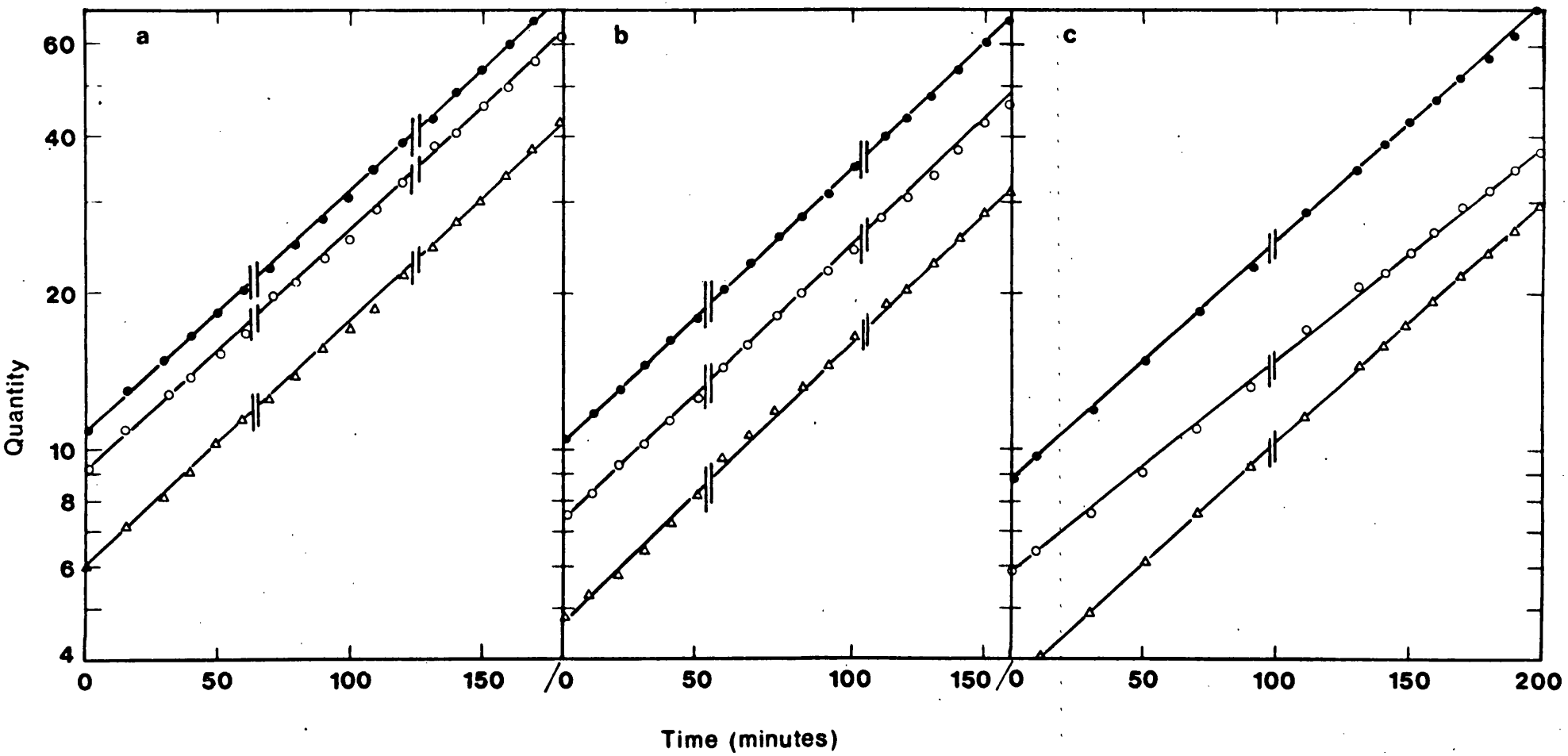
4.I. CONDITIONS FOR STEADY-STATE GROWTH

In order to study changes in the cell cycle events arising as a result of variations in the velocity of replication, it is necessary to exclude all other possible sources of perturbations to the culture. This can be achieved by ensuring that the culture maintains a balanced state of growth during the course of the experiment. Although the thymine concentration can be varied over a wide range (see section 3.II) with no detectable effect upon the rate of mass increase of LEB16, it is not correct to assume that the cultures are at all times in states of balanced exponential growth, (see for example Zaritsky & Pritchard, 1973).

In Fig. 6 it can be seen that when this strain is grown in PA medium with concentrations of thymine of 20 µg/ml or 1.5 µg/ml absorbance, particle number and DNA content all increase at the same rate. Also shown are measurements taken on a culture growing in 1.0 µg/ml thymine. Here the culture is once again in exponential growth but the growth is not balanced. Although absorbance and DNA content increase together, cell number increases at a slower rate. In this situation cell mass $\bar{M}$ continues to increase while the DNA/mass ratio $\bar{G}/\bar{M}$ remains constant. In all cases the doubling times of the cultures, as measured by A_{450}, are identical : only at even lower

Figure 6 : EFFECT OF THYMINE CONCENTRATION ON THE RATE OF INCREASE IN ABSORBANCE, CELL NUMBER AND DNA

(•) Absorbance (10^{-2} A_{450}), (o) cell number (10^7/ml) and (Δ) counts per minute (10^3/ml) in exponential, PA medium, cultures of LEB16 uniformly labelled with ^{14}C-thymine (0.05 μCi/μg) at concentrations (a) 20 μg/ml, (b) 1.5 μg/ml (c) 1.0 μg/ml. After each doubling in cell number the cultures were diluted twofold with fresh prewarmed medium. The points on the graphs have been corrected for these dilutions.


a
b
c
60
40
20
10
8
6
4
Quantity
0
50
100
150
0
50
100
150
0
50
100
150
200
Time (minutes)

thymine concentrations (see section 3.II) does the growth rate become reduced.

Although variation in exogenous thymine concentration has no effect on the rate of growth it does affect the average size of the cells and the DNA/mass ratio of the culture. As the amount of thymine is reduced the average cell mass increases and the DNA/mass ratio decreases (Fig. 7). These changes are qualitatively those to be expected if a lowering of the thymine concentration causes an increase in the length of the replication period C. Quantitative measurements of these changes are used in a later section (section 4.III) to analyse the effects of variation in the replication velocity on the cell cycle parameters C and D.

The "unbalanced" growth observed at the lowest thymine concentration (1.0 μg/ml) in PA medium is more marked at faster growth rates. In glycerol medium growth is unbalanced in 10 μg/ml thymine and the degree of unbalance is even greater in glucose medium at this thymine concentration (Fig. 8). In another thymineless mutant (*E.coli* $15T^-$) it has similarly been shown that the minimum concentration of thymine required for balanced growth increases with growth rate (Zaritsky & Pritchard, 1973).

These observations clearly demonstrate that the conditions under which meaningful measurements of cell cycle parameters of thymineless strains of *E.coli* can be made are very limited. In the experiments reported in this thesis, only those growth conditions giving balanced growth are used.

Figure 7 : DNA/MASS RATIO AND CELL MASS

DNA/mass ratio ($\bar{G}/\bar{M}$) and average cell mass ($\bar{M}$) for LEB16 grown in PA medium with thymine concentrations 20 µg/ml (•), 1.5 µg/ml (o) and 1.0 µg/ml (Δ). The values are calculated from the data in Fig. 6.

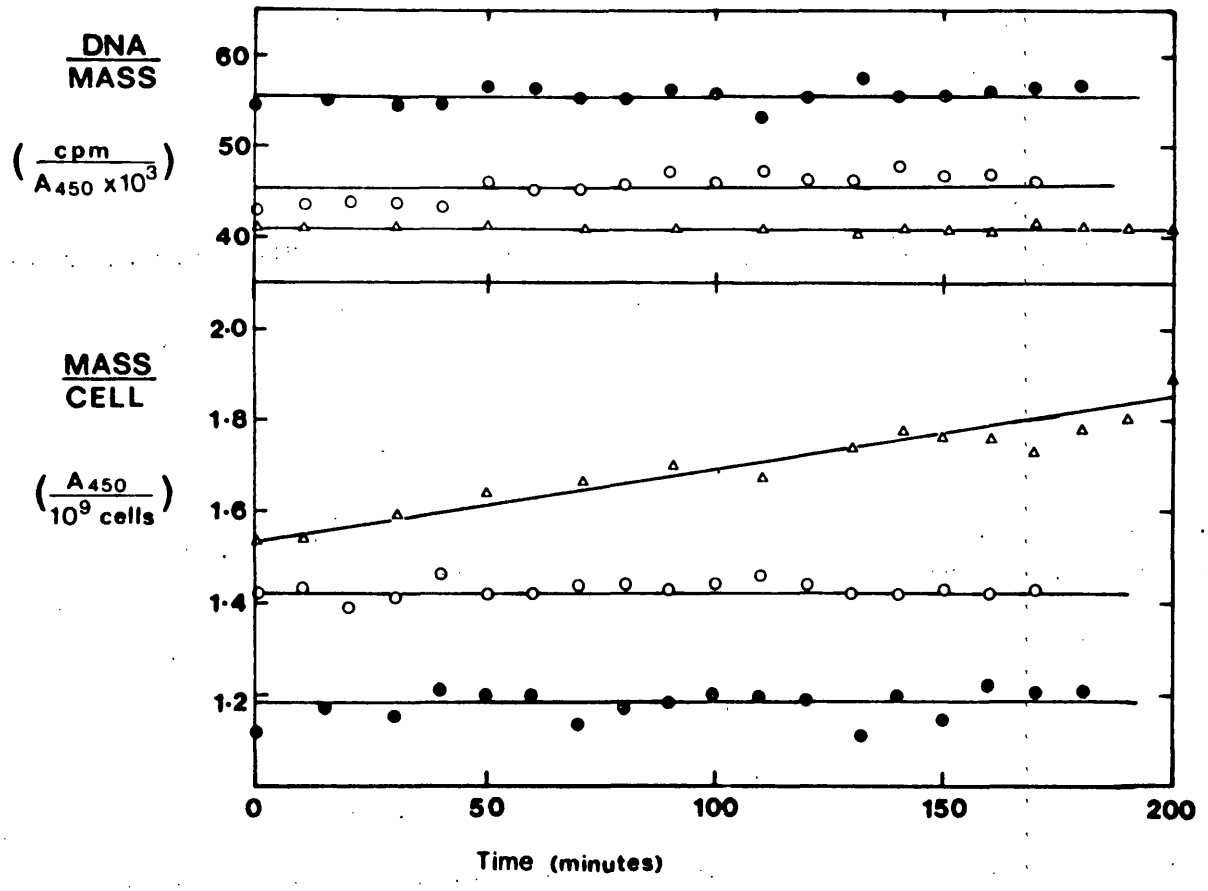
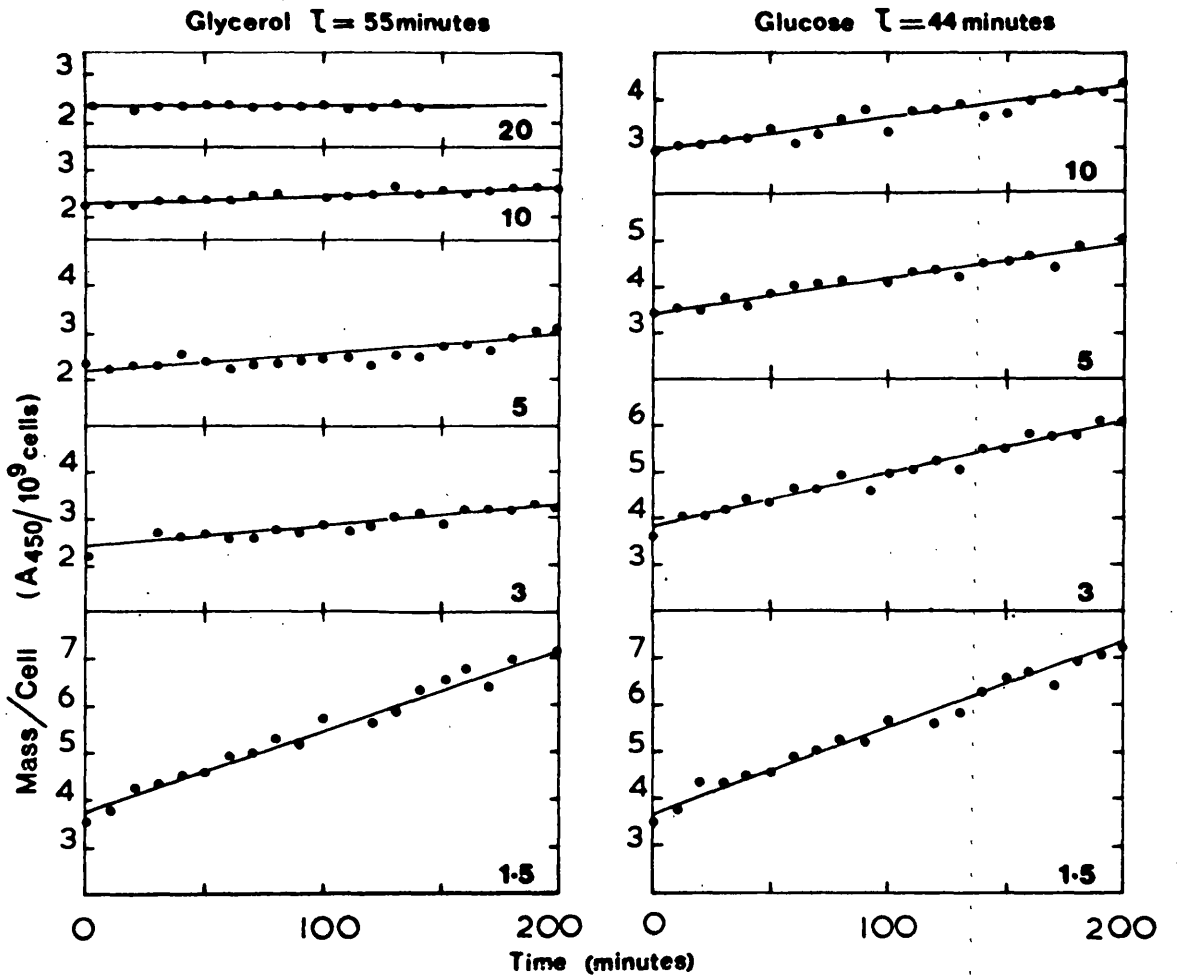

DNA
MASS
(cpm / A450 x10^3)
60
50
40
2·0
MASS
CELL
(A450 / 10^9 cells)
1·8
1·6
1·4
1·2
0
50
100
150
200
Time (minutes)

Figure 8 : EFFECT OF THYMINE CONCENTRATION ON THE RATE OF INCREASE OF CELL MASS

Measurements of cell size of LEB16 grown in media containing glucose or glycerol as carbon sources. Thymine concentration (µg/ml) are as indicated in the frames.


Glycerol τ = 55minutes
Glucose τ = 44 minutes
Mass/Cell (A450/10^9 cells)
20
10
5
3
1·5
0
100
200
Time (minutes)

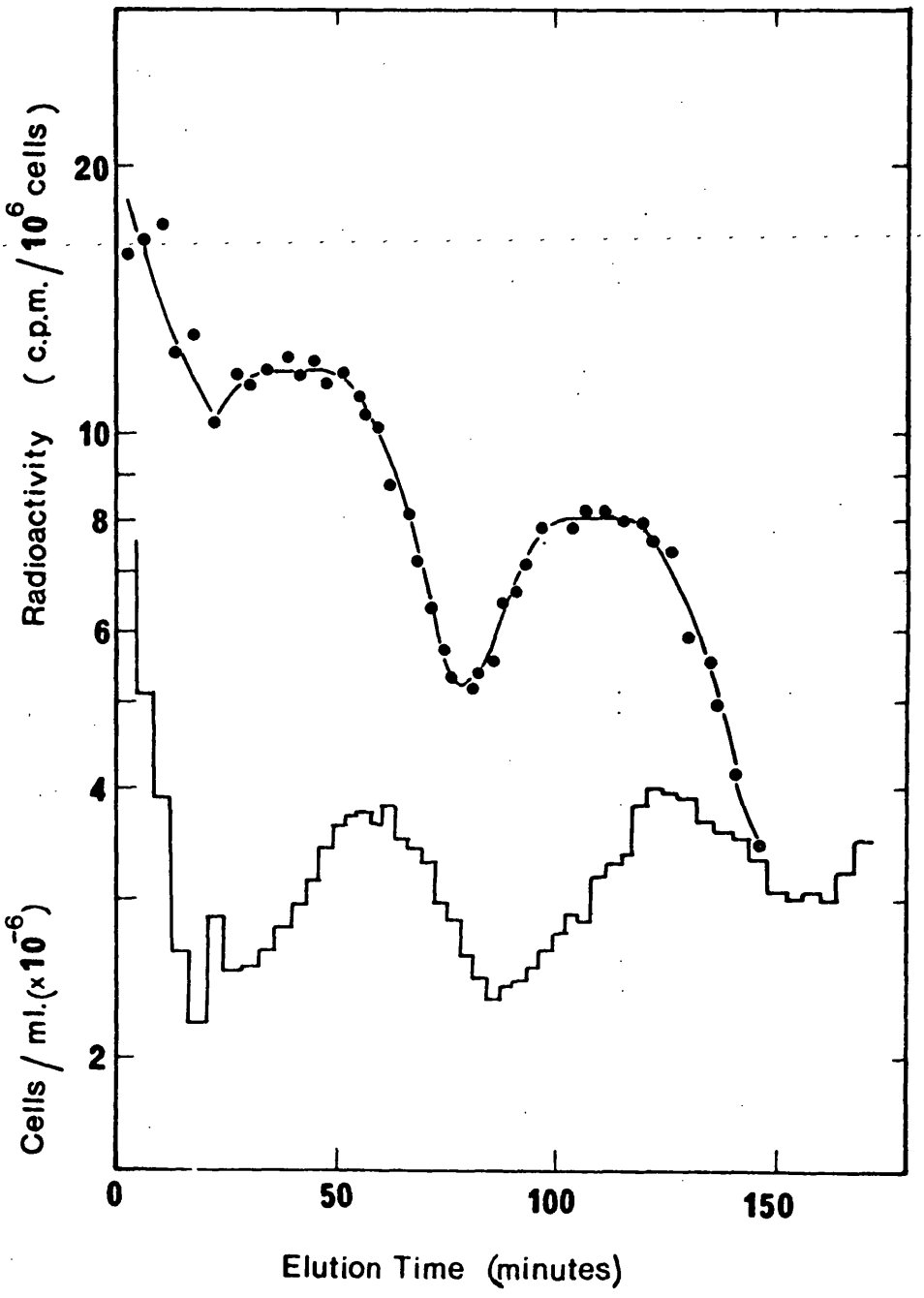
4.II. THE PATTERN OF DNA SYNTHESIS DURING THE CELL CYCLE AS A FUNCTION OF THYMINE CONCENTRATION

The pattern of DNA replication during the cell cycle and the effects produced by changes in replication velocity have been examined by the membrane elution technique. For each experiment an exponential steady-state culture, growing in a prescribed amount of thymine was briefly exposed to 14-C-thymidine (1 µg/ml; 0.25 µCi/ug) and the progeny cells fractionated according to their ages so that the radioactivity incorporated at different cell ages could be measured. The method of interpretation of this type of experiment has been outlined earlier (see Materials and Methods), and more fully discussed elsewhere (Helmstetter, 1967).

The result of an experiment performed on LEB16 in PA medium supplemented with 20 µg/ml thymine is shown in Fig. 9. In my system I find that the unbound cells take approximately 10 to 15 minutes to be completely removed from the membrane. This tends to obscure the early portion of the curve, and a clearer representation of the events occurring during the cell cycle can be obtained from consideration of the second cycle of elution, i.e. that portion of the curve between the peaks on the elution profile at 60 minutes and 120 minutes. Here it can be seen that the rate of DNA synthesis varies during the cell cycle. Initiation of rounds of chromosomal replication, indicated by the increase in radioactive content per cell, occurs at

Figure 9 : DNA SYNTHESIS DURING THE DIVISION CYCLE IN HIGH THYMINE

Plots of (•) radioactivity per effluent cell and (—) cell density in consecutive samples of the eluate from a ^{14}C-thymidine pulse-labelled, membrane-bound population of LEB16 growing in PA medium containing 20 µg/ml thymine.

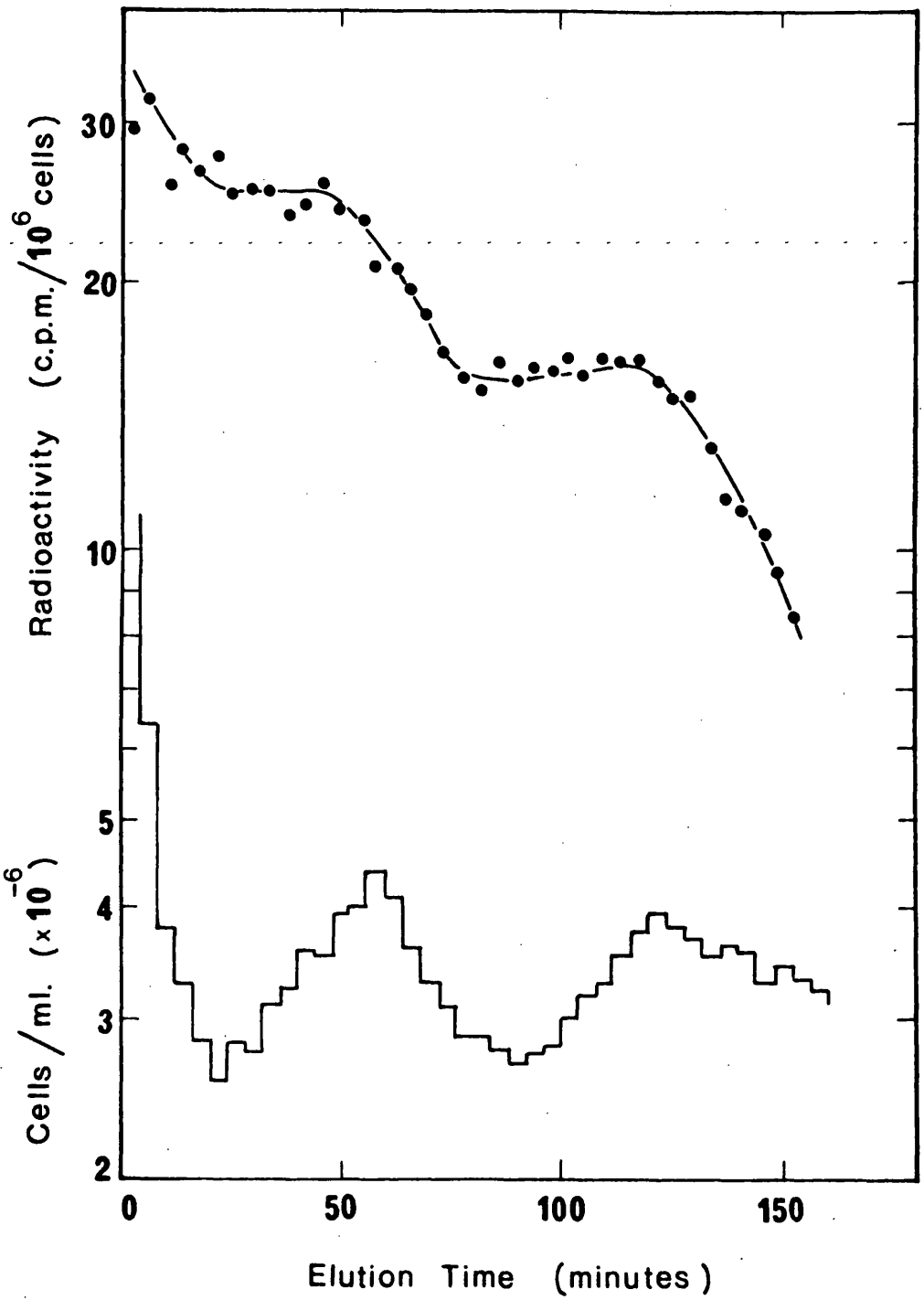

Radioactivity (c.p.m. / 10^6 cells)
Cells / ml.(x10^-6)
20
10
8
6
4
2
0
50
100
150
Elution Time (minutes)

a later cell age just prior to division. Termination, denoted by the fall in radioactive content occurs about 20 to 30 minutes before division, so that there is a gap in the cell cycle during which there is no ongoing DNA synthesis. This pattern of replication and its relationship to the division cycle agrees very well with previous estimates of the cell cycle parameters C and D of 41 and 22 minutes respectively in a wild-type E.coli B/r (Cooper & Helmstetter, 1968). Under conditions of growth in low (2.0 μg/ml) thymine the pattern of DNA synthesis is somewhat different (Fig. 10). The trough which was observed in the high thymine elution curve is now absent. Thus there is no gap between rounds of replication, and DNA synthesis occurs at all cell ages under these conditions. Notice that the profile of eluted cells is unaffected by the reduction in thymine. This supports the previous observation (section 4.I) that balanced growth is maintained at the same growth rate over this range of thymine concentration.

If, as is suggested (Pritchard & Zaritsky, 1970), lowering the thymine concentration leads to an increase in the replication time C, this should be reflected in a movement of the time at which initiation occurs relative to termination. Progressive increases in C will result in a progressive shortening of the gap between rounds of replication until C becomes equal in length to the doubling time τ when initiation and termination will coincide.

Figure 10 : DNA SYNTHESIS DURING THE DIVISION CYCLE IN LOW THYMINE

Plots of (•) radioactivity per effluent cell and (—) cell density in consecutive samples of the eluate from a ^{14}C-thymidine pulse-labelled, membrane-bound population of LEB16 growing in PA medium containing 2 µg/ml thymine.


Radioactivity (c.p.m./10^6 cells)
30
20
10
Cells / ml. (x 10^-6)
5
4
3
2
0
50
100
150
Elution Time (minutes)

DNA synthesis will then occur continuously throughout the division cycle. If C exceeds τ a period of dichotomous replication will be observed. The pattern of replication observed in 2.0 µg/ml thymine is that expected when the replication period becomes the same as the doubling time. It is similar to that observed for wild-type cells growing in glucose minimal salts medium (Clark & Maaløe, 1967; Helmstetter, 1967). Completion of the round of replication is not detected because it is obscurred by the succeeding initiation event which occurs at the same time. The disappearance of the gap between rounds of replication provides direct evidence that lowering the thymine concentration causes an increase in the replication period of LEB16.

An analysis of the manner in which the times of initiation and termination vary with increasing C should reveal what is happening to D under these conditions. If D remains constant then a progressive lengthening of C should lead to a corresponding progressive movement of the initiation event to an earlier cell age. Alternatively, an increase in C at the expense of D would result in no change in the age at which initiation occurs, but would cause a movement of termination to a later cell age. Both possibilities result in the same eventual qualitative effect, namely the coincidence of initiation and termination observed in low thymine grown cells. This is shown diagrammatically in Fig. 11.

Figure 11 : THEORETICAL PATTERNS OF DNA SYNTHESIS DURING THE DIVISION CYCLE FOR VARIOUS RELATIONSHIPS BETWEEN C AND D

The rate of DNA synthesis as a function of cell age for cultures with different replication times. The rows (a) - (d) represent increases in the length of C such that :-

$C < \tau = C+D$ (a)

$C < \tau < C+D$ (b)

$C = \tau < C+D$ (c)

$C > \tau < C+D$ (d)

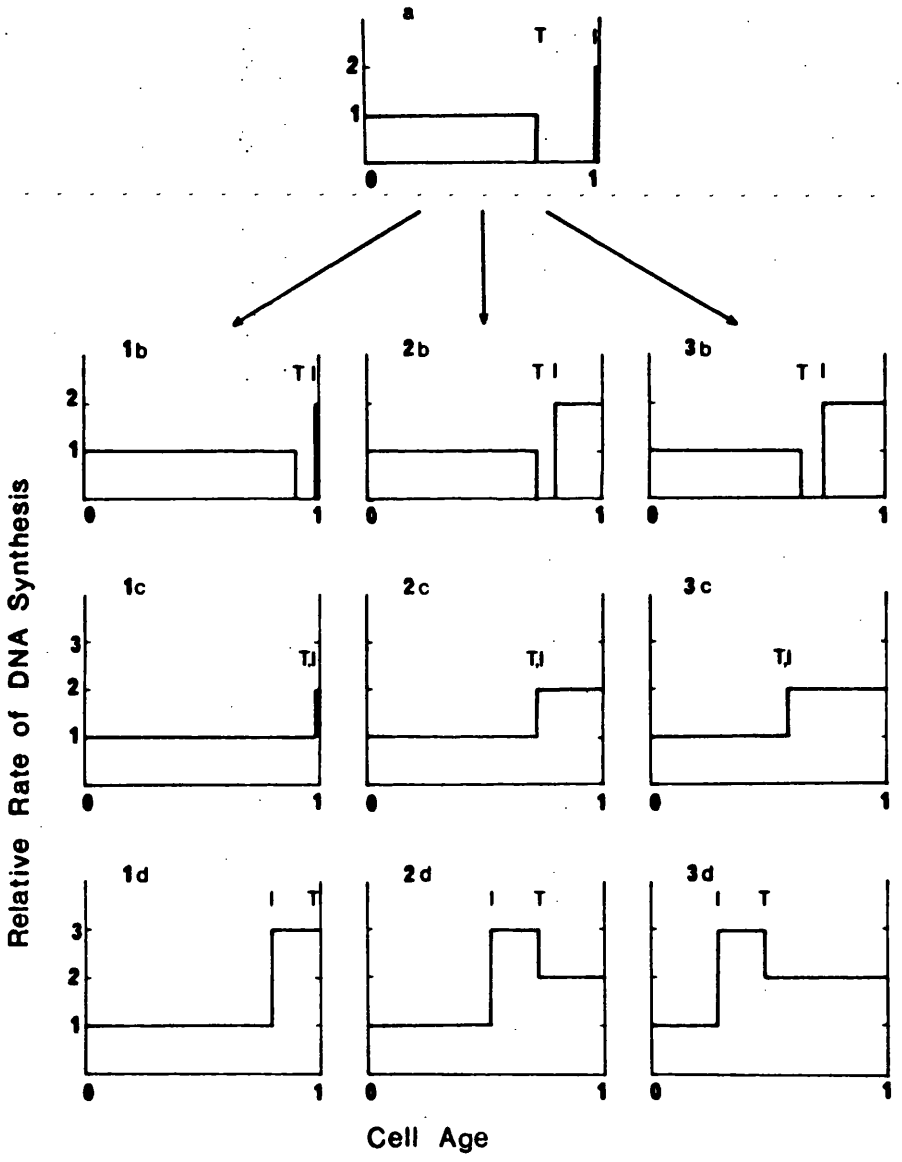
The columns (1) (2) (3) represent possible relationships between C and D :-

D decreases as C increases (1)

D remains constant (2)

D increases as C increases (3)

The time of initiation and termination are denoted in each case by the letters I and T respectively.


a
T
2
1
0
1
1b
T I
2c
T,I
3d
I
T
Relative Rate of DNA Synthesis
Cell Age

I have examined the pattern of replication during the cell cycle in a series of experiments conducted over the range of thymine concentration where the growth of LEB16 in PA medium is balanced. The results are presented in Fig. 12. Also included, is the result of an experiment performed with LEB18, the Thy$^+$ revertant of LEB16. It can be seen that the pattern of replication in both LEB18 and high thymine grown LEB16 are almost identical. This indicates that C and D in high thymine grown cells are of similar durations to the wild-type values. As the thymine concentration is gradually lowered there is a gradual disappearance of the gap in DNA synthesis.

An important point in this connection is that the variance in the population is not greatly affected by lowering the thymine concentration. The ratio of the peak to dip values from the elution profile after one generation of growth on the membrane (see Materials and Methods section 2V) remain virtually unchanged over the range of concentrations used here (Table 3). Therefore changes in the spread of the distribution over the ages at which initiation and termination take place will also be very small, and will not contribute significantly to the observed changes in pattern of DNA synthesis. However this variance over the ages of initiation and termination does create difficulties in making quantitative assessments of the lengths of C and D. In Fig. 13 the data from the second generation of the elution curves are plotted on a cell age basis. Although the peak to peak cycle has been

Figure 12 : ELUTION PATTERNS OF CULTURES GROWN IN DIFFERENT THYMINE CONCENTRATIONS

Plots of a typical profile of effluent cell density (a) and radioactivity per effluent cell (b-h) from ^{14}C-thymidine pulse-labelled, membrane-bound populations :- (b) E.coli B/r Thy^+ LEB18; (c), (d), (e), (f), (g), (h), E.coli B/r Thy^- (t1r) LEB16 grown in media containing (20), (5), (4), (3), (2), (1.5) µg/ml thymine respectively. The arrows denote the positions of the peaks of the cell density profile.

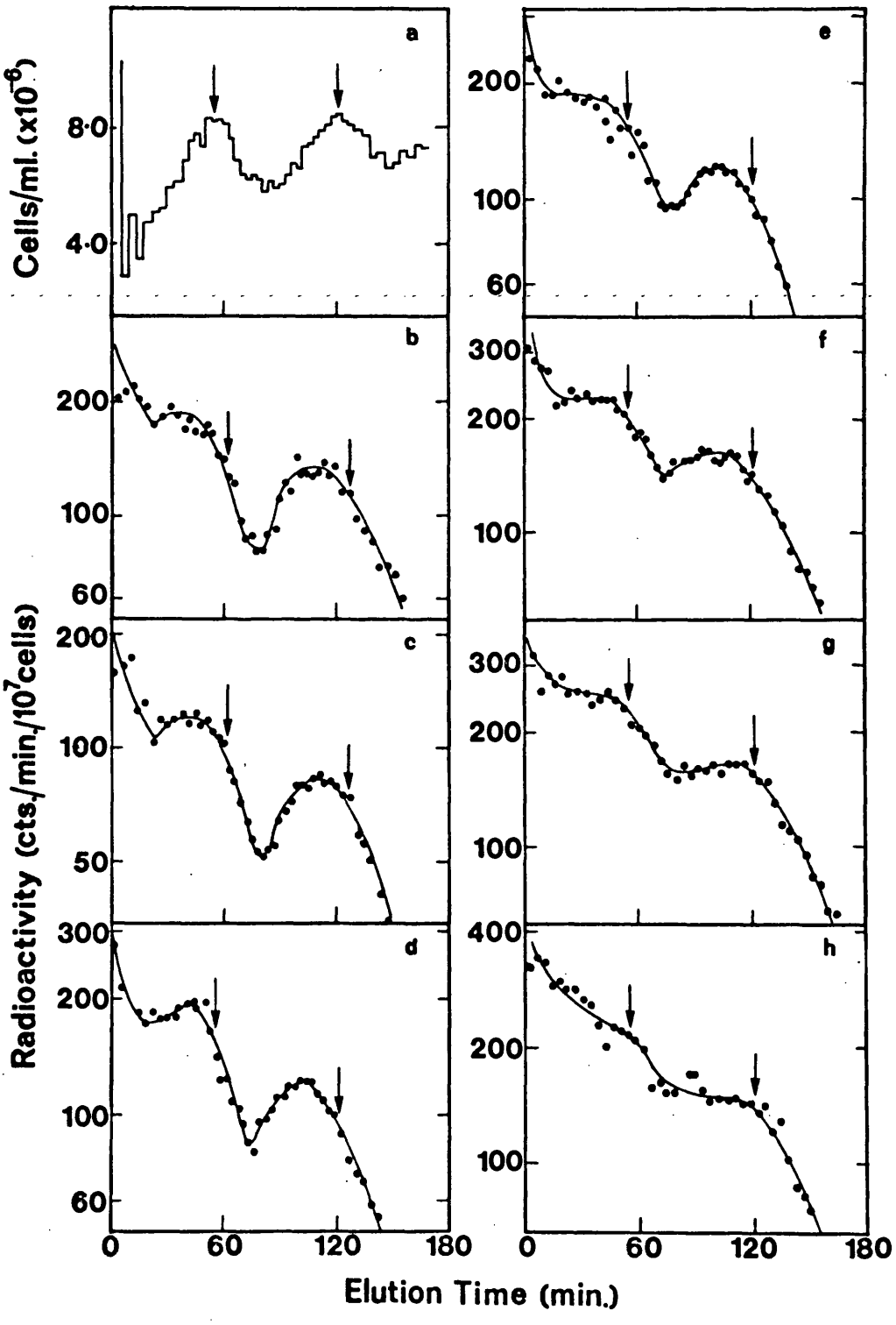

Cells/ml. (x10⁻⁶)
Radioactivity (cts./min./10⁷cells)
Elution Time (min.)
a
b
c
d
e
f
g
h
8·0
4·0
200
100
60
300
50
400
0
60
120
180

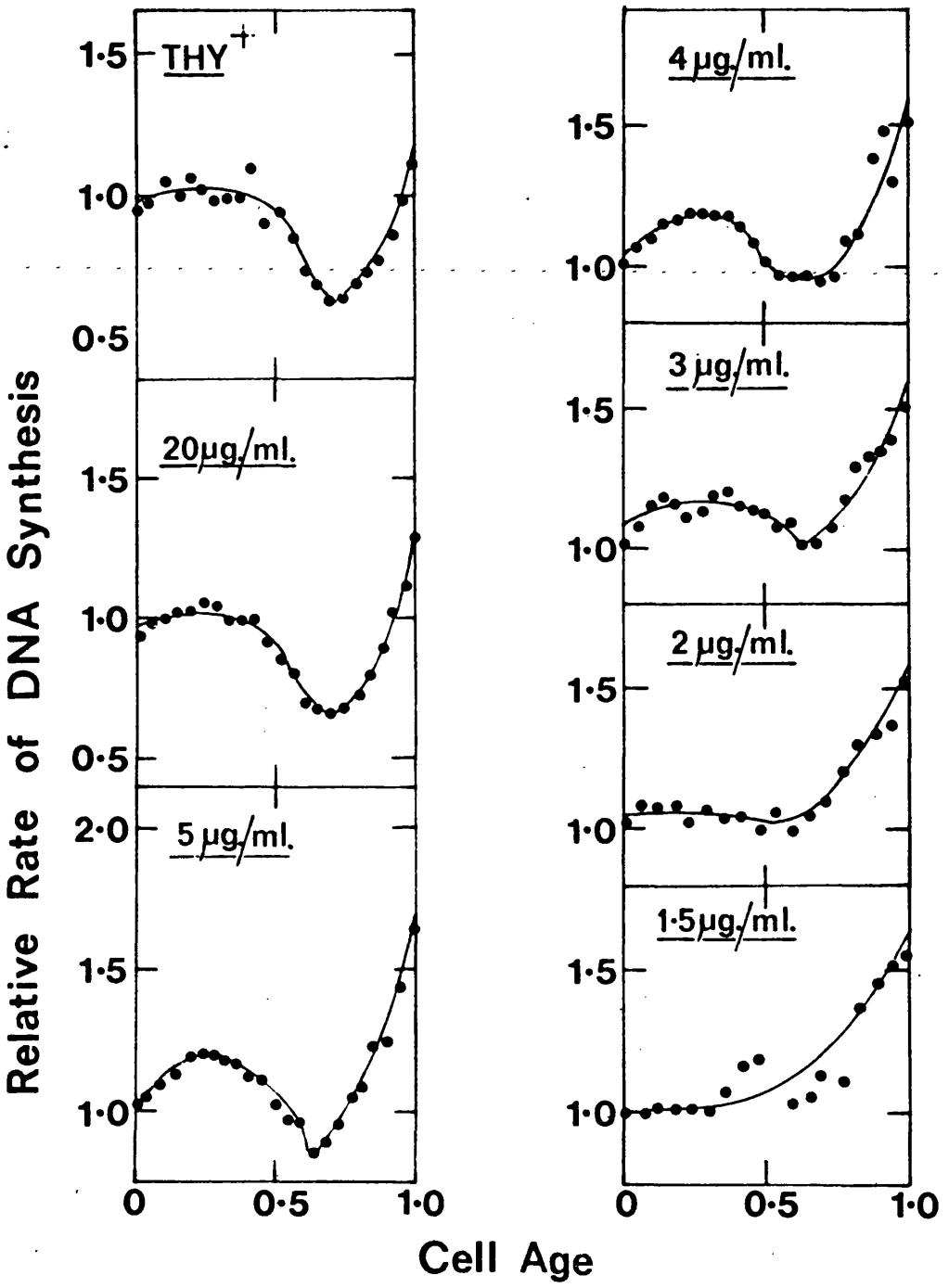
Table 3. VARIANCE IN DOUBLING TIMES OF MEMBRANE-BOUND POPULATIONS

Thymine (µg/ml)	Variance (a)
THY^+	1.7
20	1.7
5	1.65
4	1.6
3	1.9
2	1.7
1.5	1.6

(a) expressed as the ratio of Peak to Dip values after one generation of growth on membrane (see section 2, V)

Figure 13 : RELATIVE RATE OF DNA SYNTHESIS DURING THE DIVISION CYCLE AT DIFFERENT THYMINE CONCENTRATIONS

The figure is a replot, on a cell age basis, of the curves (b-h) in the second generation of elution in Fig. 12. i.e. that portion of the elution curves between the two arrows. The data have been normalised to an initial rate of DNA synthesis of 1.


THY+
20µg./ml.
5 µg./ml.
4 µg./ml.
3 µg./ml.
2 µg./ml.
1·5µg./ml.
Relative Rate of DNA Synthesis
Cell Age
0
0·5
1·0
1·5
2·0

used for this plot, and a more accurate description might have been obtained from the half height cycle (see Helmstetter & Cooper, 1968) this is not important in this context because it is the overall pattern of changes that is of interest in analysing the effects on C and D.

Unfortunately it is difficult to draw definite conclusions about the relationship between C and D from these data. Although initiation remains late in the cell cycle the data of Fig. 13 appear to show that it may be occurring a little earlier in the low thymine cells. However, this change is probably not sufficient to account for the complete disappearance of the gap between rounds of replication. It is therefore possible that an accompanying displacement of termination towards division and towards initiation, also takes place. The changes are not sufficiently large to permit a clear identification of the mode of change of C and D.

Although the levels of radioactivity per eluted cell cannot be directly compared between experiments, because of the technical difficulties in exactly controlling the pulse times and conditions, it does seem that the number of replication forks per cell is not greatly altered by growth in low thymine. If large changes in C or extra initiation events, had been produced this would have resulted in extensive periods of dichotomous (multifork) replication. These are not observed.

The fall-off of the radioactive content of the effluent cells in the third cycle of elution is an

unexpected observation. This is also observed with other E.coli B/r strains at slow growth rates (C.E. Helmstetter personal communication) and could be due to ordered segregation of DNA strands at division as described by Pierucci and Zuckowski (1973).

4.III. MEASUREMENT OF CELL SIZE AND DNA COMPOSITION AS A FUNCTION OF THYMINE CONCENTRATION

The membrane elution experiments described in the previous section demonstrate qualitatively that variations in exogenous thymine concentration cause changes in the pattern of DNA replication during the division cycle of LEB16. Since there is a considerable spread around the means of the cell ages at which events such as termination and initiation are occurring, as indicated by the general roundedness of the elution curves, it is difficult to obtain accurate estimates of the changes in C and D from this type of experiment. It therefore seems prudent that a second unrelated method which does not experience this potential source of error and provides an independent estimate of the effect of changes in C on D, should also be used. A quantitative assessment of changes in these parameters can be made from measurements of the size and DNA content of cells in exponential steady-state of growth in different thymine concentrations.

When a culture is in a balanced state of exponential growth the DNA content $\bar{G}$ and mass $\bar{M}$ of the average cell,

and the DNA/mass $\bar{G}/\bar{M}$ ratio of the culture can be described as functions of the three cell cycle parameterss C, D and τ as given by the equations (2), (3) and (4) (see Introduction). If the initiation mass k (Donachie, 1968) is unaffected by variation in C (Chandler & Pritchard, 1975; Pritchard & Zaritsky, 1970; Zaritsky & Pritchard, 1973) then the changes in C will lead to variations in $\bar{G}$, $\bar{M}$ and $\bar{G}/\bar{M}$. The magnitude of these changes will in the case of $\bar{G}$ and $\bar{M}$ but not of $\bar{G}/\bar{M}$, depend upon the value of D for each increase in C. Therefore by comparing variations in these three parameters, with predictions calculated from the equation for different C values, it should be possible to deduce whether there is any alteration to the length of the D period.

Table 4 shows the results of a series of experiments in which DNA/mass, cell mass and DNA content have been measured at different thymine concentrations over the range of conditions where growth is balanced. It is clear that reduction in the amount of thymine causes a progressive decrease in DNA/mass ratio and an increase in average cell size. There appears to be very little effect upon the average DNA content per cell. These changes are qualitatively those to be expected if a lowering of the thymine concentration causes an increase in the length of C.

From these data it is possible to determine whether there is any effect upon D. Inspection of the equations (2), (3), (4) shows that whereas $\bar{G}$ and $\bar{M}$ are functions of

Table 4. AVERAGE CELL SIZE AND COMPOSITION AT DIFFERENT THYMINE CONCENTRATIONS

Thymine µg/ml	DNA MASS c.p.m. x 10^{-3} / A_{450}	C [a] min.	MASS CELL predicted [b]	MASS CELL observed	DNA CELL predicted [b]	DNA CELL observed
20	58.64 ± 0.28	41	1.00	1.00	1.00	1.00
5	55.97 ± 0.50	51 ± 3	1.11	1.07 ± 0.01	1.06	1.01 ± 0.01
4	53.47 ± 0.37	60 ± 3	1.22	1.08 ± 0.02	1.12	0.99 ± 0.01
3	52.79 ± 0.34	64 ± 3	1.28	1.15 ± 0.02	1.14	1.03 ± 0.02
2	51.47 ± 0.47	68 ± 3	1.33	1.16 ± 0.02	1.17	1.02 ± 0.01
1.5	49.81 ± 0.59	75 ± 3	1.44	1.24 ± 0.02	1.22	1.04 ± 0.02

(a) Calculated from changes in DNA/mass ratio as in Pritchard and Zaritsky (1970).

(b) Calculated according to equations (2) and (3) using C values from column 3 and assuming D = 22 min.

C, D and τ, $\bar{G}/\bar{M}$ is independent of D. Since the doubling time (τ) is unaffected over this range of thymine concentrations the $\bar{G}/\bar{M}$ data can be used to quantitate changes in C. The results of these calculations, which are made by a ratio method (Pritchard & Zaritsky, 1970) based upon C of 41 minutes in 20 µg/ml thymine (see membrane elution data) are presented in Table 4. I have also calculated predicted values for $\bar{M}$ and $\bar{G}$ for each value of C, assuming that D remains constant at 22 minutes. Comparison of the predictions and observations indicates that a discrepancy exists between the two sets of data. It could be that the assumption about the constancy of D is incorrect. It is impossible to choose a single value for D such that the predictions and observations about $\bar{M}$ and $\bar{G}$ are equivalent at all thymine concentrations.

These results can be used further to explore the relationship between C and D. I have used the measurements of average cell mass $\bar{M}$ to quantitate changes in C+D arising as a result of the variation in C. By substraction of the estimates of C which have already been calculated (Table 4), it is possible to arrive at an estimate of the size of D in each steady-state condition (Table 5). Calculations of this type, show that as the transit time (C) for a replication fork is extended there is a shortening of the period between termination and division. The absolute values of C and D obtained by this type of analysis are dependent upon the values chosen for the base

Table 5. EFFECT OF THYMINE CONCENTRATION ON THE LENGTH OF D

Thymine μg/ml	C [a] min.	MASS CELL $\frac{A_{450}}{10^9 \text{ cells}}$	C+D [b] min.	D [c] min.
20	41	1.23 ± 0.01	63	22
5	51 ± 3	1.31 ± 0.01	68 ± 2	17 ± 5
4	60 ± 3	1.33 ± 0.01	70 ± 2	10 ± 5
3	64 ± 3	1.41 ± 0.01	75 ± 2	11 ± 5
2	68 ± 3	1.43 ± 0.01	77 ± 2	9 ± 5
1.5	75 ± 3	1.52 ± 0.01	82 ± 2	7 ± 5

(a) From Table 4. (b) Calculated from changes in average mass per cell using the relationship:-

$$(C+D)_2 = \left[\frac{\gamma . \log \left(\frac{\bar{M}_2}{\bar{M}_1}\right)}{\log 2}\right] + (C+D)_1$$

derived from equation (3). (c) Calculated by subtracting (a) from (b).

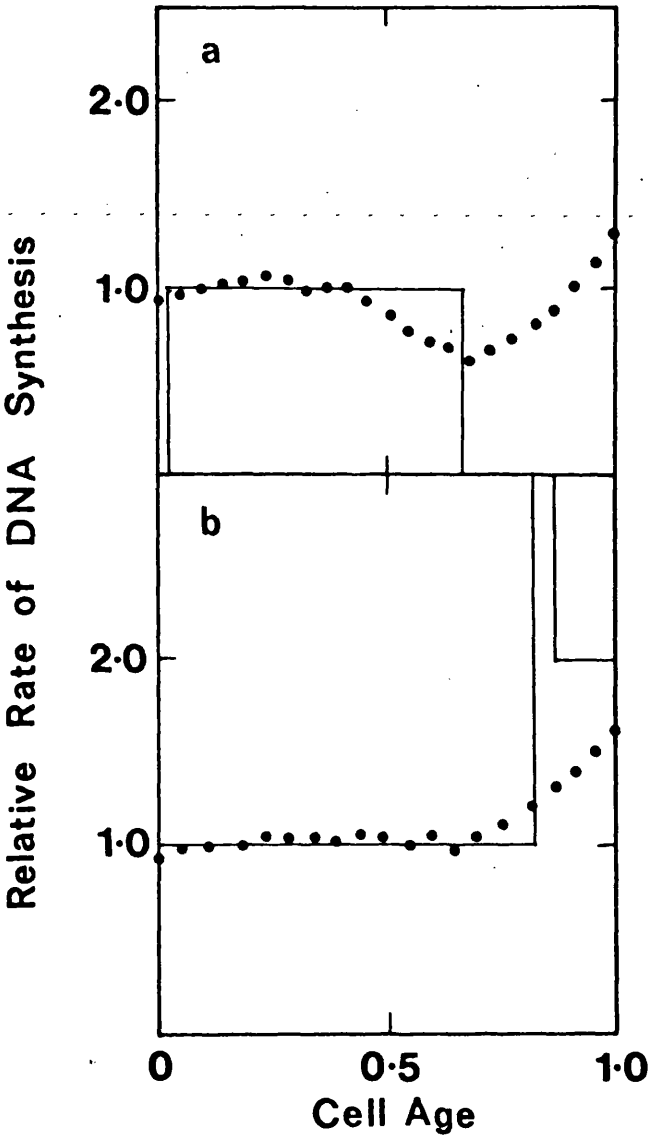
level; in this case C and D have been assumed to be equal to the wild-type values of 41 and 22 minutes respectively in high (20 μg/ml) thymine (see membrane elution data, section 4.II). However, the size of the changes in C and D are independent of values chosen. Unfortunately, due to the slow growth rate used in these experiments, the changes observed in $\bar{M}$ and $\bar{G}/\bar{M}$ are quite small. Thus the random error in sampling results in quite large ranges for each calculated value of D (Table 5). As with the elution data, the validity of the absolute values in D calculated in this way is less important than the general trend of changes.

The data of Table 5 appear to show that the changes in C and D are not equivalent. The increment in C is larger than the decrease in D. Thus examination of the pattern of DNA synthesis during the division cycle should show changes in the time of occurrence of both initiation and termination. Fig. 14 shows a comparison between the observed pattern of DNA synthesis in high (20 μg/ml) and low (2 μg/ml) thymine (membrane elution data, Figs. 9, 10) and the cycle predicted from the values of C and D in Table 5. The similarity indicates a correlation between the two sets of data.

These results qualitatively lend support to the model that the time of cell division is measured from the time of initiation of rounds of replication so that D is merely a difference between the duration of the division and replication processes. However, the inequality of

Figure 14 : OBSERVED AND PREDICTED PATTERNS OF DNA SYNTHESIS DURING GROWTH IN HIGH AND LOW THYMINE

Observed rate of DNA synthesis (•) during the cell cycle for cultures in (a) 20 μg/ml and (b) 2 μg/ml thymine (data from Figs. 9 and 10 plotted as in Fig. 13). Theoretical rate of DNA synthesis (—) calculated from the values of C and D in Table 4.


a
b
2·0
1·0
2·0
1·0
Relative Rate of DNA Synthesis
0
0·5
1·0
Cell Age

the changes in C and D suggests that even this is too simple an interpretation of the data. The data do not agree with the postulate that the D period represents a process taking a fixed time under all growth conditions.

A more general form of these two models with which the data of Table 2 are consistent, is that the sequence of events leading to division takes a fixed length of time and is triggered by an event in the replication cycle occurring Cx minutes after initiation, x being the fraction of the replication time between initiation and the division signal. Here D is the period between completion of this process $C(1-x)+D$ and the time $C(1-x)$ taken to replicate the remaining portion of the chromosome. Thus a reduction in replication velocity $(\frac{1}{\Delta C})$ will lead to a delay (ΔCx) in the occurrence of the division signal, and a corresponding delay, because of the constancy of the division process, in the time of division. The models suggesting division is triggered either at initiation or termination are extreme forms of this general model where x has the values 0 and 1 respectively.

By taking pairwise combinations of the data for C and D at different replication velocities and solving for x in the equation

$$C_1 (1-x) + D_1 = C_2 (1-x) + D_2 \quad \ldots\ldots\ldots (6)$$

an average value for x of about 0.6 is obtained. Therefore if there is a division sequence of fixed duration the steady-state analysis indicates that it is initiated just

over half way through the replication cycle. If the wild-type value of 41 and 22 minutes for C and D are taken with the calculated value of x then insertion of these values into the term $C(1-x)+D$ shows that the division process has a duration of 38 minutes.

It is perhaps not out of place to speculate on how such a control mechanism might function. One possible method could be that the division sequence is triggered by the product of a particular gene which is only expressed at the time of its replication. Just such a mode of gene expression has previously been suggested for the production of an inhibitor of replication initiation (Pritchard *et al.* 1969) and more recently evidence has been published indicating this as the possible method of regulation of synthesis of the lactose operon repressor (Edelman & Edlin, 1974). In the situation described here the fractional replication time x of the gene, will correspond to the fractional distance of the gene from the chromosome origin. On the assumption that replication proceeds bidirectionally, in a symmetical manner, from an origin located at 74 minutes on the Taylor map (Bird *et al.* 1973), calculations show that the gene would be situated at either map position 47 minutes or 11 minutes. It may be significant in this context, or merely coincidental with these speculations, that the mutations *min* and *lon* both of which lead to aberrant cell division, map very close to one of these *loci*.

5. CHROMOSOME REPLICATION AND THE REGULATION OF CELL DIVISION

5.I. EFFECT OF CHANGING REPLICATION VELOCITY ON THE TIME OF CELL DIVISION

Investigations by other workers (Clark, 1968a,b; Helmstetter & Pierucci, 1968; Dix & Helmstetter, 1973) have shown that there is a specific relationship between replication and cell division. Inhibition of DNA synthesis prior to completion of a round of replication leads to an inhibition of cell division, whereas the same treatment applied after termination has no such effect. It has been concluded from these findings that termination of a round of replication is a necessary condition for cell division and may determine the time at which it occurs.

The results of the previous section are in qualitative agreement with the hypothesis that a coupling exists between replication and division. A reduction in replication velocity results in an increase in the time (C+D) between initiation of a round of replication and the subsequent cell division. If division is timed from an event in the replication cycle, other than initiation, then a reduction in replication velocity at the time of initiation should lead to a delay in the time of cell division. The extent of the delay will be equal to the difference between C+D arising from the change in replication velocity.

When LEB16 is grown in PA medium with high thymine (20 μg/ml) initiation of a round of chromosome replication

occurs at the time of cell division (see membrane elution data, section 4.II). Thus by collection of the newborn cells from a membrane-bound culture a population of cells can be obtained which is synchronised at this stage of the division cycle. In the experiment shown in Fig. 15 half of such a population was resuspended in PA medium with low (2 µg/ml) thymine. The other half was maintained in high thymine medium as a control. The two cultures were incubated and their cell densities determined at suitable time intervals.

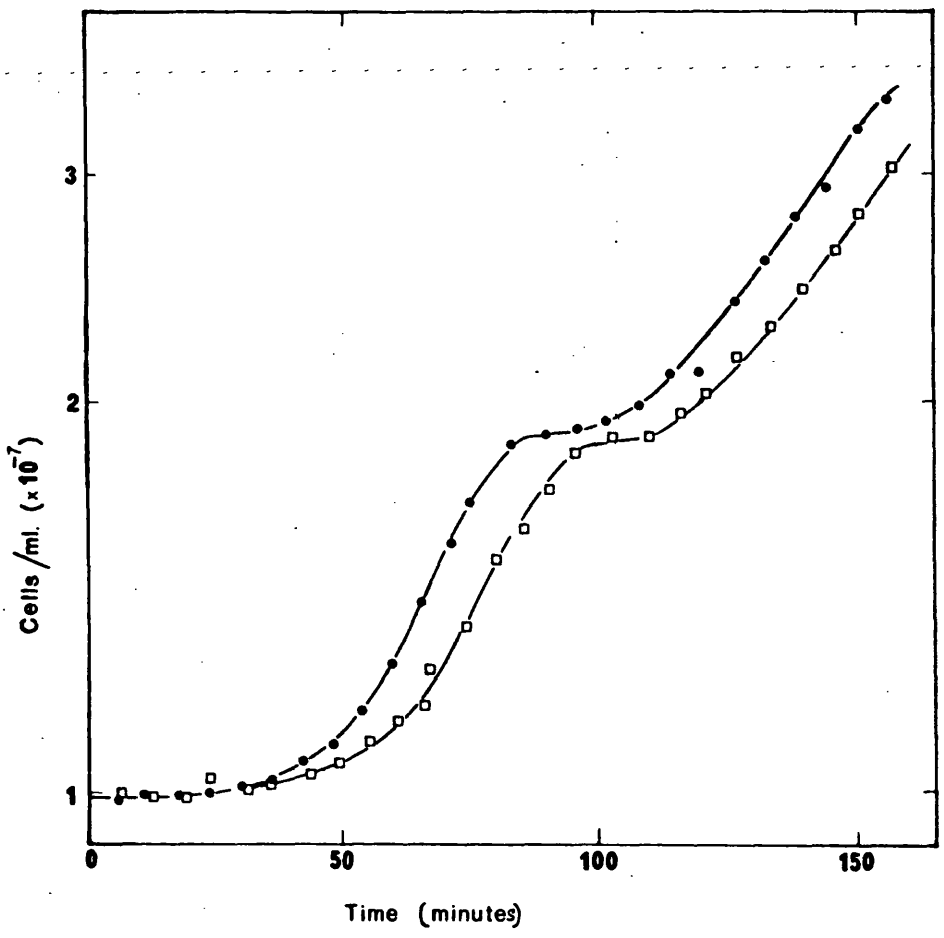
The result obtained (Fig. 15) shows that division is delayed when the culture is grown in low thymine i.e. with a slow replication velocity. The extent of the delay, although difficult to estimate accurately because of the distribution of division times, appears to be in the order of 10 to 15 minutes. This is in good agreement with the difference of 14 minutes between estimates of C+D in high and low thymine calculated from the change in average cell mass (Table 5).

5.II. THE KINETICS OF TRANSITIONS BETWEEN DIFFERENT STEADY-STATES OF GROWTH

The ability to vary replication velocity can be used in another way to investigate the temporal relationship between the chromosome replication cycle and cell division. For when a steady-state exponential culture growing in high thymine is transferred to medium with a lower thymine concentration there is a transient reduction in the rate

Figure 15 : CELL DIVISION AFTER A REDUCTION IN REPLICATION VELOCITY

Plots of cell density during synchronous growth of newborn cells collected from a membrane-bound population grown in 20 µg/ml thymine, and resuspended in (•) 20 µg/ml or (□) 2 µg/ml thymine.

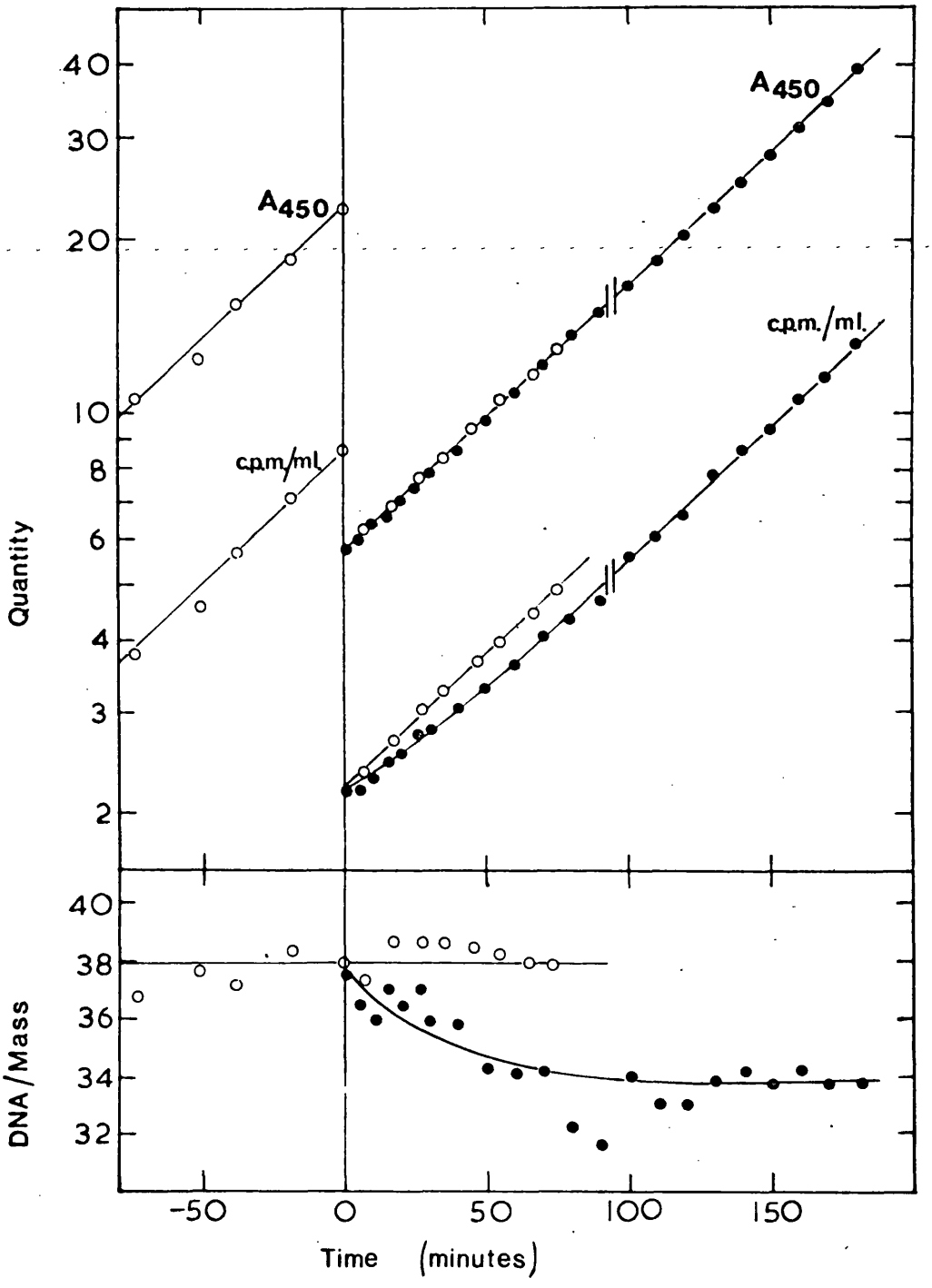

3
2
1
Cells /ml. (x10⁻⁷)
0
50
100
150
Time (minutes)

of DNA synthesis which results in a decrease in the DNA/mass ratio of the culture. This is due to the reduction in the velocity of travel of forks along the chromosome and hence delay in their time of arrival at the chromosome terminus. Eventually when the distribution of replication forks equilibrates to that appropriate for the new replication velocity the previous rate of synthesis is re-established. By definition the time taken for this transition is equal to the new replication time. Fig. 16 shows the result of an experiment in which LEB16 was transferred from 6 µg/ml to 1.5 µg/ml thymine. As predicted there is an immediate reduction in the rate of DNA synthesis. The normal rate is regained about 70 to 80 minutes after the transition in accordance with the C time of 75 minutes for this thymine concentration (Table 4).

It follows from this that if division is timed from an event in the replication cycle, other than initiation, it too will be transiently delayed after a "step-down". This has been shown in the synchronous culture experiment, (Fig. 15). However, any cells which have already started on the sequence of events leading to division, at the time of the transition will be unaffected by the change in replication velocity and will continue to progress to their normal division. Only in the following cell cycle will their division time be altered by the reduction in the replication velocity. For example, if division is timed from termination as has been suggested, then there should be a delay of D minutes before the alteration in the rate of termination is reflected by a change in the rate of cell division. If division is timed from an earlier

Figure 16 : KINETICS OF INCREASE OF ABSORBANCE AND DNA AFTER A REDUCTION IN REPLICATION VELOCITY

Absorbance (10^{-2} A_{450}) and counts per minute (10^3/ml) in a uniformly labelled culture of LEB16 growing in 6 µg/ml ^{14}C-thymine (0.03 µCi/µg) (0). At 0 min the culture was diluted fourfold into fresh prewarmed medium containing, (0) the same concentration of ^{14}C-thymine (0.03 µCi/µg) or (•) no thymine to give a final concentration of 1.5 µg/ml. At 95 min the latter culture was diluted twofold into prewarmed medium containing the same concentration of ^{14}C-thymine (0.03 µCi/µg) to maintain A_{450} below 0.2. The points after this time are corrected for the dilution. The lower section of the figure shows the same data plotted as DNA/mass ratio ($\left[cpm/A_{450}\right] \times 10^{-3}$).


40
30
20
10
8
6
4
3
2
A450
A450
c.p.m./ml.
c.p.m./ml.
Quantity
40
38
36
34
32
DNA/Mass
-50
0
50
100
150
Time (minutes)

event, at chromosomal age x, then the delay (C(1-x)+D) will be correspondingly longer. In fact the length of the delay will be equal to the duration of the division process. As in the case of DNA synthesis following a step-down, the normal steady-state rate of division will be re-established in a time equal to the new C+D after the transition.

Fig. 17 shows the result of a step-down experiment in which LEB16 was transferred from 6 μg to 1.5 μg thymine. Division continues at the pre-step rate for approximately 20 minutes and then decreases, which results in an increase in cell size since the rate of mass synthesis (A_{450}) is unchanged. The new steady-state is reached about 80 minutes after the transition. These kinetics are precisely those predicted if a terminal event of the replication cycle times cell division.

In the complementary step up experiment (Fig. 18) there is an acceleration in the rate of cell division. In this situation the delay between the transition and the change in division rate is short (less than 10 minutes). If the delay period represents the length of D in the pre-step conditions these kinetics are consistent with the earlier findings that a lengthening of C is accompanied by a decrease in D.

It should perhaps be pointed out that the result from the "step-down" experiment (Fig. 17) apparently differs from that, previously reported from this laboratory, for a similar experiment performed on E.coli 15T$^-$ (Zaritsky & Pritchard, 1973). These authors were unable to detect a continuation of the pre-step rate of division following

Figure 17 : KINETICS OF INCREASE OF ABSORBANCE AND CELL NUMBER AFTER A REDUCTION IN REPLICATION VELOCITY

Absorbance ($10^{-2}A_{450}$) and cell number (10^7/ml) in a culture of LEB16 growing in 6 µg/ml thymine (o). At 0 min the culture was diluted fourfold into fresh prewarmed medium containing, (o) the same concentration of thymine or (•) no thymine to give a final concentration of 1.5 µg/ml. At 125 min the latter culture was diluted twofold with prewarmed medium containing the same concentration of thymine, to maintain A_{450} below 0.3. The points after this time are corrected for the dilution. The lower section of the figure shows the same data plotted as average cell mass ($A_{450}/10^9$ cells).

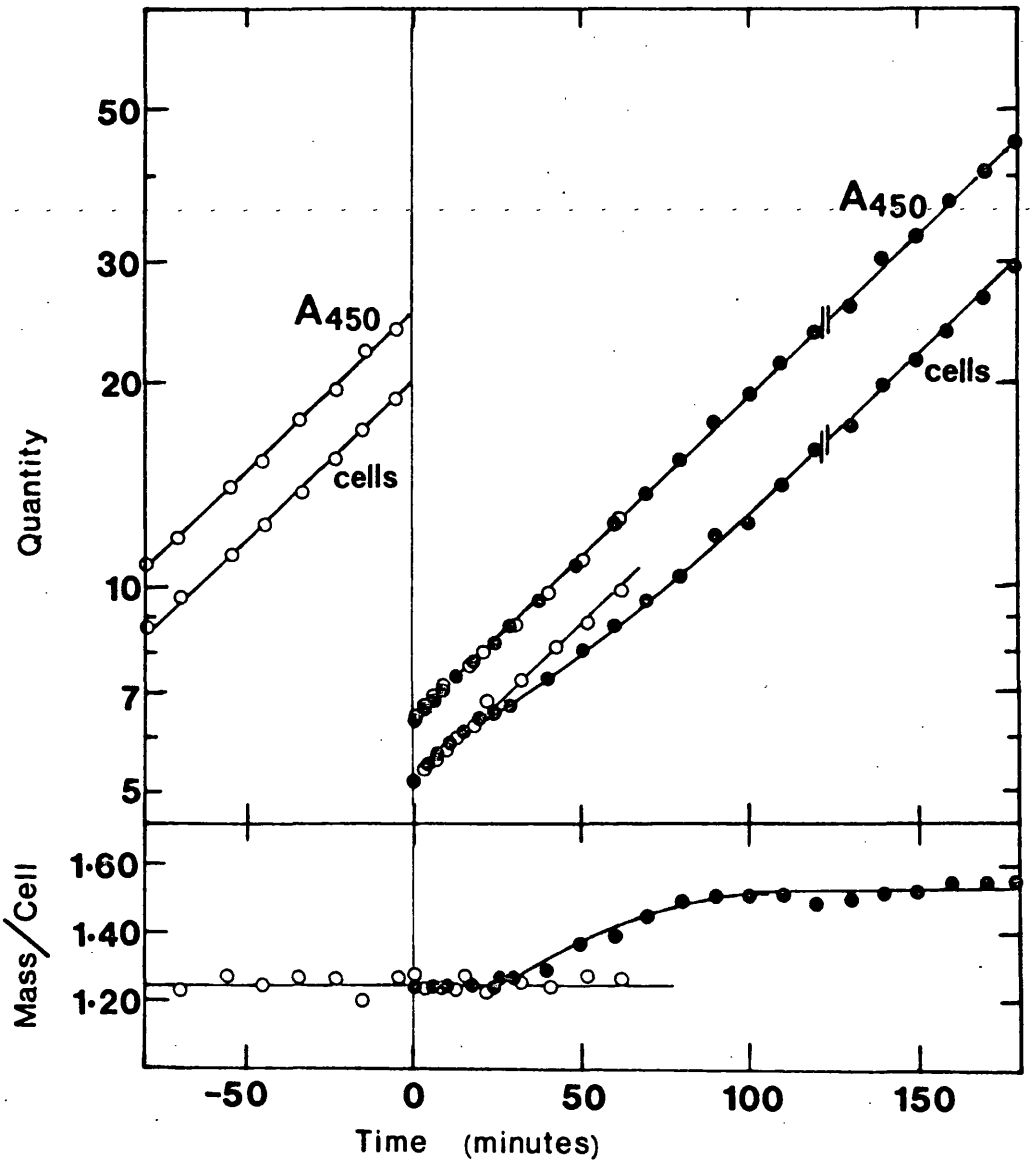
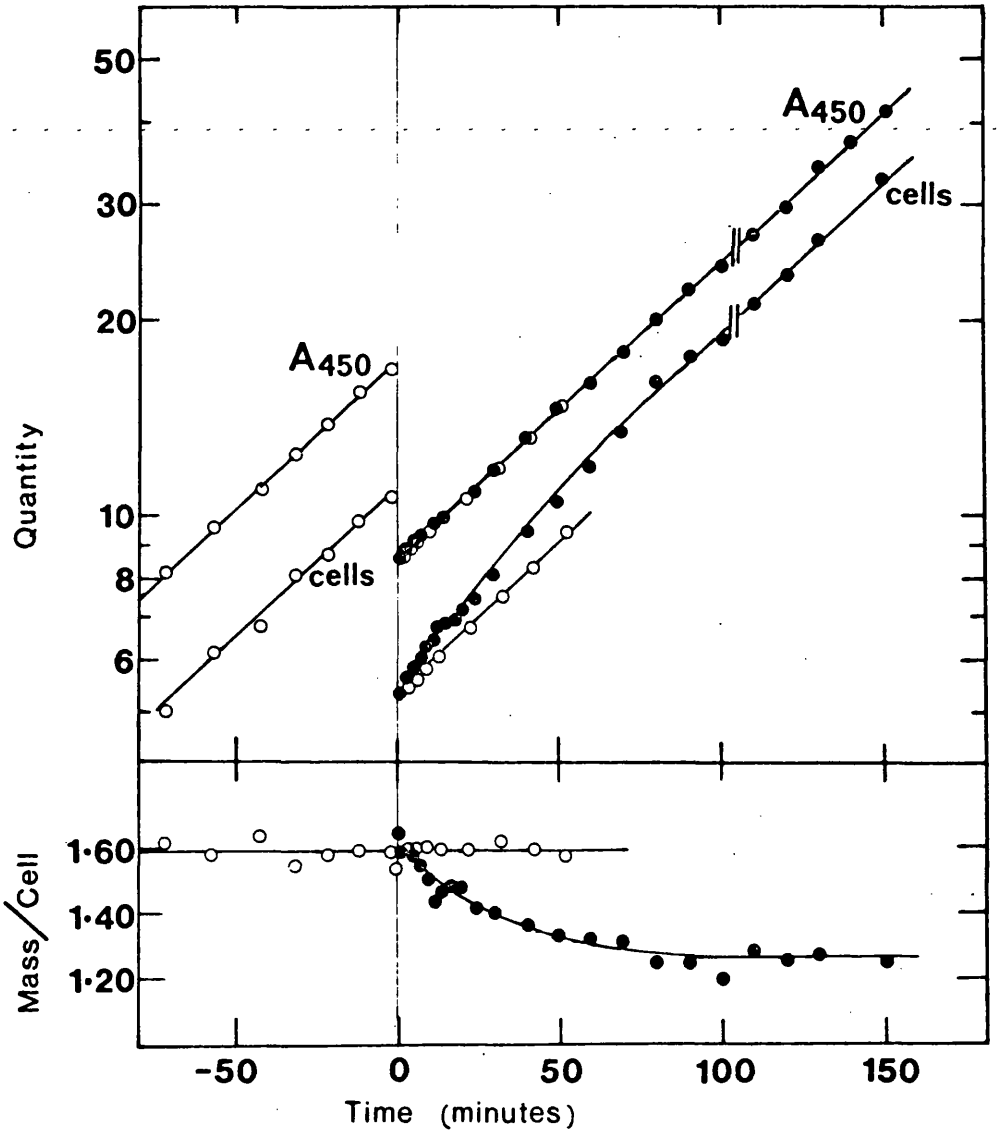

50
30
20
10
7
5
Quantity
A450
cells
A450
cells
1·60
1·40
1·20
Mass/Cell
-50
0
50
100
150
Time (minutes)

Figure 18 : KINETICS OF INCREASE OF ABSORBANCE AND CELL NUMBER AFTER AN INCREASE IN REPLICATION VELOCITY

Absorbance (10^{-2} A_{450}) and cell number (10^7/ml) in a culture of LEB16 growing in 1.5 µg/ml thymine (o). At 0 min the culture was diluted twofold into fresh prewarmed medium containing, (o) the same concentration of thymine, or (•) 11 µg/ml thymine to give a final concentration of 6.25 µg/ml. At 105 min the latter culture was diluted twofold with prewarmed medium containing the same concentration of thymine, to maintain A_{450} below 0.3. The points after this time are corrected for the dilution. The lower section shows the same data plotted as average cell mass ($A_{450}/10^9$ cells).

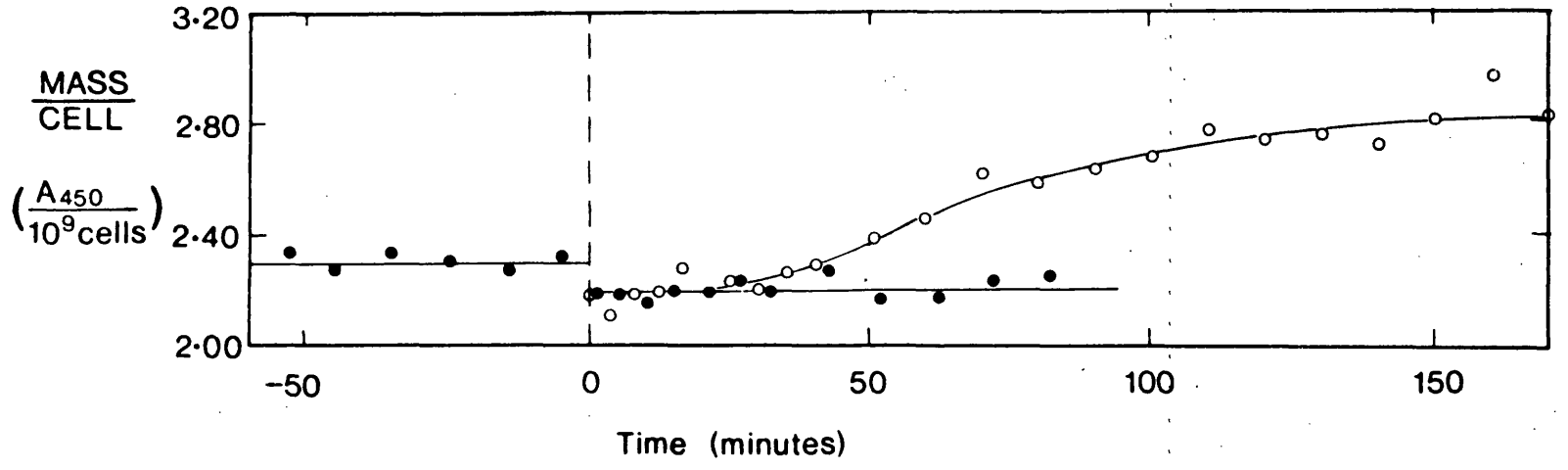

50
30
20
10
8
6
Quantity
A450
cells
A450
cells
1·60
1·40
1·20
Mass/Cell
-50
0
50
100
150
Time (minutes)

transfer to a lower thymine concentration. I have repeated their experiment (Fig. 19) using the same strain, P178, in which I have carefully examined the rate of division immediately after the transition. My results show that the pre-step cell size, and therefore rate of division, is maintained for a period of about 20 minutes after the shift. Apart from this difference the shape of the curve is very similar to that obtained by Zaritsky and Pritchard (1973). The apparent discrepancy may be due to the fact that previously insufficient samples were assayed immediately following the thymine shift for a firm conclusion to be reached about any possible effect on cell size. I conclude from my data that both E.coli B/r T^- and E.coli 15T^- probably behave similarly under these conditions.

These experiments indicate that in steady-state exponential growth conditions the time of cell division is determined by a terminal event of the replication cycle. Thus an apparent paradox exists between these data and those that I have already described which support the hypothesis that the time of cell division is triggered earlier in the cell cycle. The data suggest that termination of a round of chromosome replication signals an act of cell division to occur D minutes later. However the time interval D between these two events is not invariant but is dependent upon the replication time C.

Figure 19 : AVERAGE CELL SIZE OF E.COLI 15T⁻ DURING A REDUCTION IN REPLICATION VELOCITY

P178 was grown in glycerol medium containing thymine at (•) 2.0 μg/ml and supplemented with required amino acids, arginine, methionine and tryptophan at 50 μg/ml. At 0 min the culture was diluted fourfold with fresh prewarmed medium containing, (•) the same concentration of thymine, or (o) no thymine to give a final concentration of 0.4 μg/ml.


MASS
CELL
(A450 / 10^9 cells)
3·20
2·80
2·40
2·00
-50
0
50
100
150
Time (minutes)

5.III. CELL DIVISION AFTER INHIBITION OF CHROMOSOME REPLICATION

The results of the transition experiments which strongly implicate termination in the determination of the time of cell division, together with the earlier observations on the relationship between C and D lead to a further prediction which is easily tested. Under conditions of an immediate inhibition of DNA synthesis the only cells in an exponential culture that are able to divide are those which had entered the D period by the time the inhibition was applied (Helmstetter & Pierucci, 1968). If therefore, termination commits a cell to divide D minutes later then the increment in cell number of an exponential population following such treatment will be dependent upon the replication time of the culture. Division should continue longer in cultures with a short replication time than in those supporting a longer replication time. The extent of this residual division is dependent upon the length of the D period and will be equivalent to the proportion of the population in that particular period of the division cycle.

There are several procedures by which DNA synthesis can be inhibited in bacterial cultures, the simplest being by removal of supplied thymine from the growth medium of a thymineless mutant. This technique was not chosen for the experiments described here because it has been shown that the size of the intracellular thymidine triphosphate pool is dependent upon the exogenous thymine concentration in such mutants (Beacham *et al.* 1971). It is possible that variations in this pool size could lead to differing amounts

of DNA synthesis and subsequent division following removal of the thymine from the medium. For the purpose of this study I have used the antibiotic nalidixic acid which has been shown to have a preferential inhibitory effect on DNA synthesis (Goss, Deitz & Cook, 1965). The overall effects of this agent appear to be similar to other treatments which also inhibit DNA synthesis (Clark, 1968a,b; Goss, Deitz & Cook, 1964; Helmstetter & Pierucci, 1968).

The effect of nalidixic acid on the growth of LEB16 in high and low thymine is shown in Fig. 20. In both cases mass synthesis (A_{450}) continues exponentially for about one doubling and then stops. Cell division continues for a shorter period and the extent of this residual division (ΔN) is dependent upon the thymine concentration. There is less division in the culture which has a longer replication time by virtue of its growth on low thymine. The results are qualitatively as predicted.

By use of the age distribution equation for an exponential population (equation 1) I have calculated a theoretical relationship for the proportion of the cell population which is in the D period (see Footnote).

Footnote: The fraction of cells f(x), in an exponential population, between the ages x_1 and x_2 is derived from the age distribution theorem, equation (1) (Powell, 1956):-

$$f(x) = \frac{\ln 2 \int_{x_2}^{x_1} 2^{(1-x)} \cdot dx}{\ln 2 \int_{0}^{1} 2^{(1-x)} \cdot dx}$$

$$= \left[- 2^{(1-x)} \right]_{x_2}^{x_1}$$

P.T.O.

Insertion of appropriate values for D and τ gives a quantitative estimate for any growth condition. Table 6 shows the measured increments in cell number (ΔN) after nalidixic acid treatment to cultures of LEB18 and LEB16 grown in various thymine concentrations. Also presented are the estimates of the proportion of the cell population in the D period under each growth condition. These calculations are based on values of D taken from Table 5. Comparison of the two sets of data shows that under all conditions the measured increment in cell division is equivalent to the fraction of the population in D.

These results are consistent with the hypothesis that termination of a round of replication is a necessary condition for cell division and commits the cell to that division after a period of D minutes.

Footnote Continued:

Thus the proportion of cells which are in the D period, f(D), is given by:-

$$f(D) = \left[- 2^{(1-x)} \right]_{x = \frac{\tau - D}{\tau}}^{x = 1}$$

$$f(D) = 2^{D/\tau} - 1$$

Figure 20 : INCREASE OF ABSORBANCE AND CELL NUMBER AFTER INHIBITION OF DNA SYNTHESIS

(•) Absorbance (10^{-2} A_{450}) and (□) cell number (10^7/ml) in culture of LEB16 growing in (a) 20 µg/ml thymine and (b) 2 µg/ml thymine to which nalidixic acid (15 µg/ml) was added at 0 min.

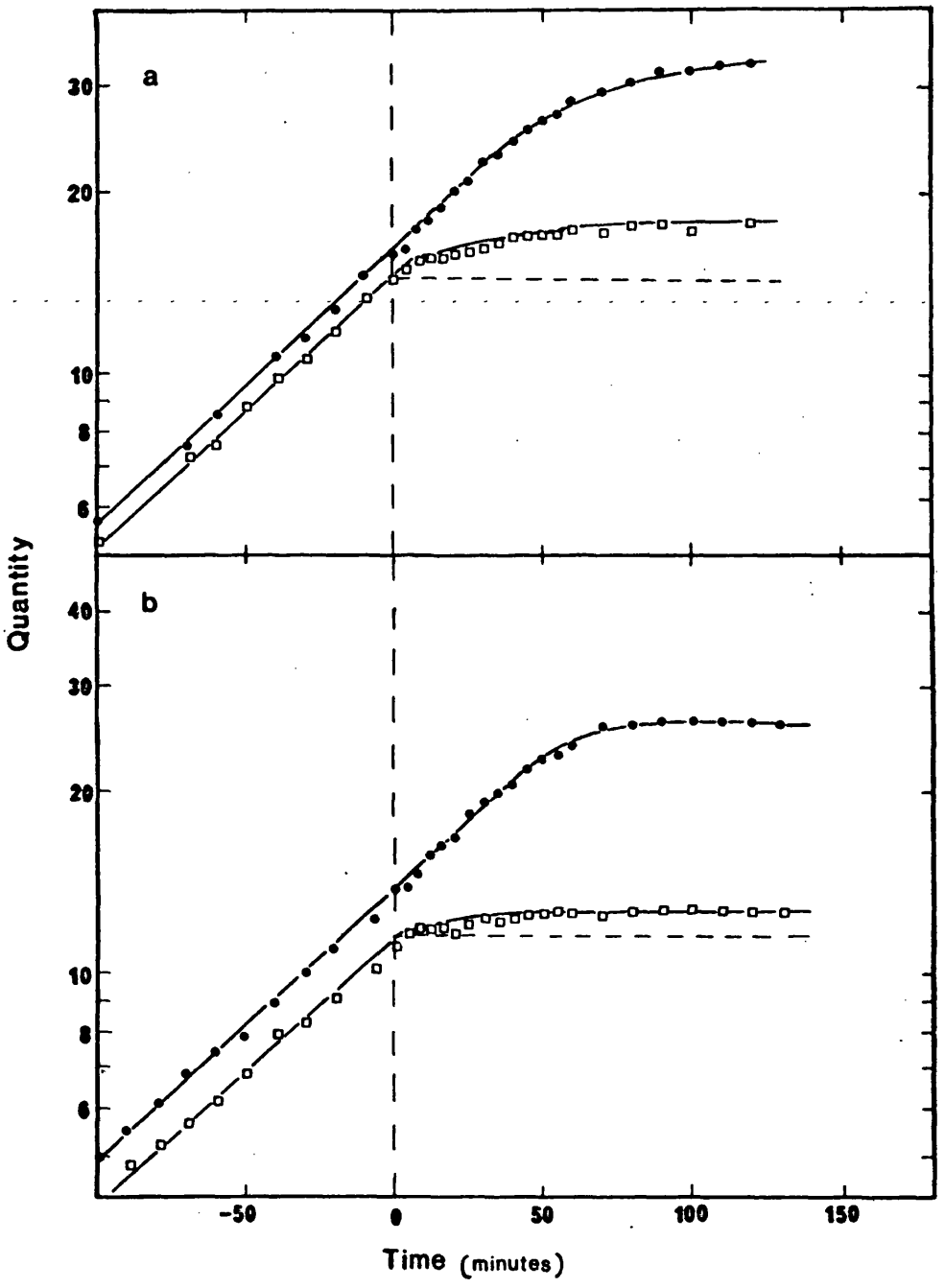

a
b
30
20
10
8
6
40
30
20
10
8
6
Quantity
-50
0
50
100
150
Time (minutes)

Table 6. INCREMENT IN CELL NUMBER AFTER INHIBITION OF DNA SYNTHESIS

Thymine (μg/ml)	Increment in Cell number (%)	D (a) (min)	Proportion of population (b) in D (%)
Thy+	22	22	27
20	22	22	27
2	13	9	10
1.5	9	7	8

(a) From steady-state data, Table 5

(b) Calculated from $f(D) = (2^{D/\tau} - 1) \times 100$ (see Footnote P.77)

6. SYNTHESIS OF OTHER MACROMOLECULES DURING THE CELL CYCLE

An alternative to the "temporal sequence" relationship for the control of cell division by chromosome replication has been offered, (see Higgins & Shockman, 1971; Previc, 1970; Pritchard, 1974). The suggestion is that division occurs as a consequence of an exponentially increasing cell mass and a linearly increasing cell envelope. The rate of synthesis of the envelope being subject to control by the replication cycle such that there is a discrete doubling in rate once per generation.

In order to investigate this proposal I have attempted to measure the rates of synthesis of cell wall murein and cell mass as a function of cell age.

6.I. THE RATE OF CELL MASS SYNTHESIS

Although there is some uncertainty about the exact manner of increase, it is generally agreed that in the majority of bacteria the rate of cell mass synthesis increases during the division cycle, (Collins & Richmond, 1962; Marr et al. 1969; Schaecter et al. 1962). Since the major proportion of the bacterial cell mass is composed of protein (Maaløe & Kjeldgaard, 1966) the rate of mass increase will largely reflect the overall rate of synthesis of this macromolecular species. Ecker and Kokaisl (1969) have examined the rate of protein synthesis by measuring grain counts as a function of cell length in autoradiographs of cells pulse-labelled with radioactive

leucine. Their findings indicate that the rate of protein synthesis increases in proportion to cell length.

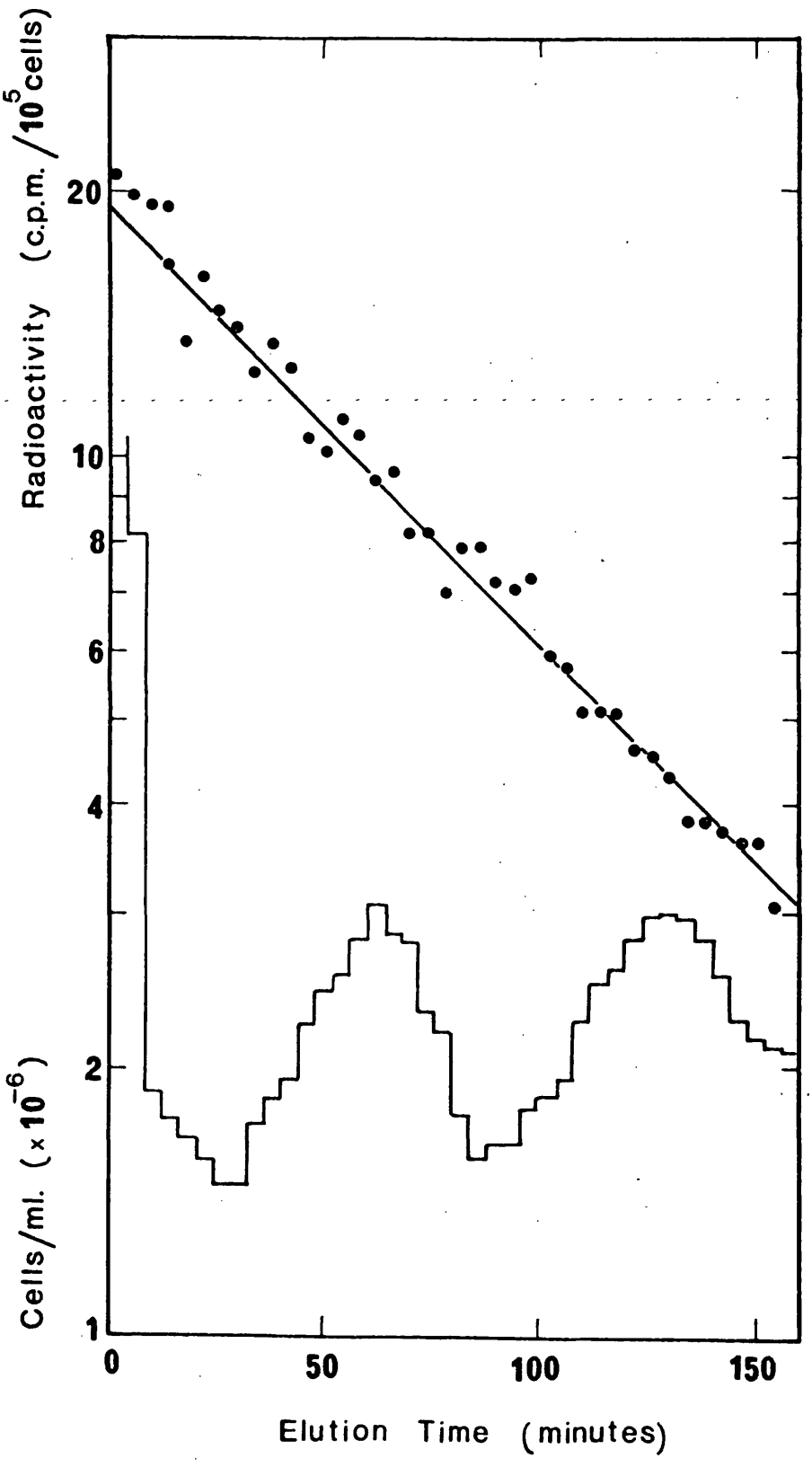
In this study I have used the membrane elution technique to measure the rate of total protein synthesis during the cell cycle. This method has an advantage over that employed by Ecker and Kokaisl in that it allows the measurements to be made as a function of cell age. In addition the technique is immune from the subjective errors in estimating grain counts and ascribing cells to "size classes" which limit the resolution of light microscopy. Pulse-labelling with ^{14}C-leucine (0.1 µg/ml, 0.233 µCi/µg) and subsequent age fractionation shows that the rate of protein synthesis increases during the cell cycle of LEB16 grown in PA medium (Fig. 21). The result confirms the observation of Ecker and Kokaisl as the data obtained fit closely to a line denoting an exponential increase in rate. In this connection it is of interest to note that a recent analysis of the differential rate of ribosome synthesis (αr) during the cell cycle has led to the conclusion that the true rate of protein synthesis is given by a complex function which deviates only 3% from a true exponential increase (Zaritsky & von Meyenburg, 1974).

6.II. THE RATE OF CELL WALL SYNTHESIS

The model set out by Pritchard postulates a linear rate of envelope synthesis with a doubling in rate once per generation. An observation of this type has been reported by Hoffman *et al.* (1972). These authors find

Figure 21 : PROTEIN SYNTHESIS DURING THE DIVISION CYCLE

Elution patterns of (•) radioactivity per effluent cell and (—) cell density in consecutive samples of the eluate from a ^{14}C-L-leucine pulse-labelled, membrane-bound, population of LEB16 growing in PA medium with 20 µg/ml thymine.


Radioactivity (c.p.m. /10^5 cells)
20
10
8
6
4
2
1
Cells/ml. (×10^-6)
0
50
100
150
Elution Time (minutes)

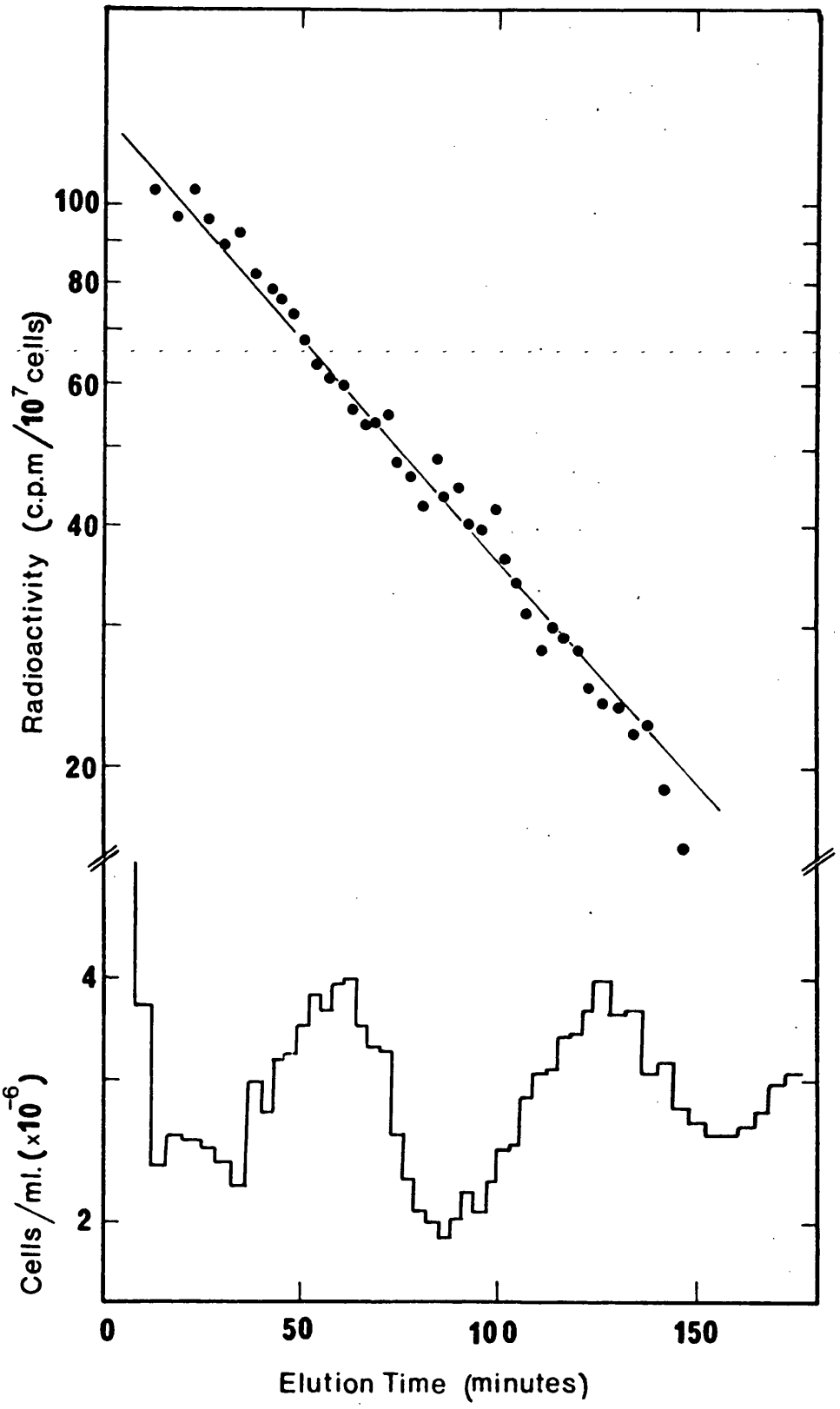
that pulse-labelling a glucose grown synchronous culture of E.coli B/r with radioactive D-glutamic acid, which is almost exclusively incorporated into cell wall murein, does produce a step pattern. The step, indicating a doubling in the rate of incorporation, occurs in mid cell cycle but cannot be unambiguously linked with a specific stage of the replication cycle as the strain used was a thymineless mutant and the pattern of DNA synthesis was not measured. Nevertheless this observation is particularly interesting because murein constitutes the rigid shape-maintaining component of the cell envelope (Schwarz et al. 1969).

I have performed a similar experiment using LEB16 with the added addition of investigating whether the doubling in rate, if detected, is associated with the replication cycle. If this is so then the time of the step should be influenced by treatments which primarily affect DNA synthesis. For instance a reduction in replication velocity should cause a delay in the time at which the step occurs if it is dependent upon replication of a specific part of the chromosome. Furthermore an inhibition of DNA synthesis prior to the step should inhibit it completely. An analysis of this nature should allow a conclusion to be reached as to whether murein biosynthesis is directly or indirectly influenced by DNA replication.

The rate of murein synthesis was measured by membrane binding and elution fractionation of ^{14}C-D-glutamic acid (1.5 µg/ml, 0.168 µCi/µg) pulse-labelled exponentially growing cells. The result obtained, Fig. 22, clearly differs from that reported by Hoffman et al. The data fit

Figure 22 : CELL WALL SYNTHESIS DURING THE DIVISION CYCLE

Elution patterns of (•) radioactivity per effluent cell and (—) cell density in consecutive samples of the eluate from a ^{14}C-D-glutamic acid pulse-labelled, membrane-bound population of LEB16 growing in PA medium with 20 µg/ml thymine.


100
80
60
40
20
Radioactivity (c.p.m /10^7 cells)
4
2
Cells /ml. (x10^-6)
0
50
100
150
Elution Time (minutes)

well with an exponential increase in rate similar to that observed for protein synthesis. There is no indication of a step pattern.

Several factors could account for this discrepancy. Firstly, an underlying assumption of the interpretation of results from membrane elution experiments is that the labelled macromolecule is stable and not subject to "turnover". In the case of B.subtilis, it has been shown that cell wall murein undergoes extensive turnover (Mauck & Glaser, 1970; Mauck, Chan & Glaser, 1971). No such observation has been recorded for E.coli (Lugtenberg & de Haan, 1971) but pulse chase experiments of Ryter et al. (1973) indicate that there may be a spatial, if not biochemical reorganisation mechanism operating after de novo biosynthesis. Any turnover resulting in loss of radioactivity from the labelled macromolecule during growth of the cells on the membrane would cause a more rapid fall in radioactive content per eluted cell than the twofold change over the doubling time expected in results obtained by this method. The radioactive content per eluted cell in Fig. 22 decrease by a factor of 2.3 over the course of one generation of growth, thereby indicating that there is no extensive turnover of the labelled material.

A second factor which could influence the incorporation pattern of the D-glutamic acid is the choice of carbon source for the growth medium; PA medium was used in the experiment described here. The related organism S.typhimurium is known to utilise proline, when supplied as sole carbon source, by conversion firstly to glutamate

and then by subsequent metabolism via the tricarboxylic acid cycle (Maaløe & Richmond, 1962). Even if the enzymes involved in the catabolism of glutamate were in a de-repressed or activated state due to growth on proline it is unlikely that the radioactive label from the supplied glutamate would be metabolised to other cellular components. Such a conversion would involve the loss of the carbon-1 atom in the decarboxylation of α-keto-glutarate to produce succinate. As the glutamic acid used in this study was labelled solely at the carbon-1 position the radioactivity would be lost as carbon dioxide. More likely is the possibility that the glutamate synthesised from proline may compete with the labelled compound as substrate for the glutamate-adding enzyme involved in murein biosynthesis. Under such conditions the incorporation pattern may not be a true reflection of the actual rate of synthesis.

Finally, it is uncertain whether there is a glutamate racemase enzyme in E.coli. If an enzyme of this nature were present then the radioactive D-glutamate may be converted to the L-isomer and subsequently incorporated into cellular protein. Investigations with a recently isolated D-glutamate auxotroph of E.coli B/r by Lugtenberg et al. (1973) have failed to detect any such activity. However, the authors were unable to find any other mechanism for the synthesis of D-glutamic acid.

In order to gain some clarification of these uncertainties I have used the sensitivity of various macromolecules to particular enzymes as a means of

investigating the site of D-glutamate incorporation. Exponential cultures of LEB16 were grown in PA medium and pulse-labelled with either ^{14}C-D-glutamic acid or ^{14}C-L-leucine. The labelled cells were subjected to the enzymatic treatment of Lugtenberg and de Haan (1971) as described in Method 2 of the Cell Fractionation procedures (Materials and Methods, section 2.VII). The amount of radioactivity remaining in macromolecular material after each stage of the procedure is shown in Table 7. Some degradation of both glutamate and leucine labelled material occurs during the pre-treatment. In the case of the glutamate labelled material, cell wall murein, this is probably due to the action of autolytic enzymes (Weidel et al. 1963). The radioactive leucine is exclusively incorporated into protein as shown by its susceptibility to trypsin digestion but not to the action of lysozyme. In contrast a significant proportion of the radioactive glutamate is converted to low molecular weight material by lysozyme indicating that it is largely incorporated into cell wall murein under the conditions used. This is in agreement with the finding of Hoffman and co-workers who report that 80% of the radioactive D-glutamate incorporated during their experiment can be released by lysozyme treatment.

In order to eliminate the possible complicating factor of endogenously synthesised glutamate affecting the pattern of incorporation I have repeated the experiment using a different carbon source which is not metabolised directly through glutamate. In glycerol medium containing 20 µg/ml

Table 7. DISTRIBUTION OF RADIOACTIVITY IN CELL FRACTIONS AS DETERMINED BY ENZYME SENSITIVITY

Step		TCA precipitable activity			
		^{14}C-D-Glutamic acid[(a)]		^{14}C-L-Leucine[(a)]	
		cts/min	%	cts/min	%
Cell suspension		1042	100	76297	100
Sonication		872	84	69380	91
100°C for	3 min	857	82	70586	93
(i) + Lysozyme	15 min	444	43	65812	86
	30 min	359	34	63418	83
	60 min	323	31	66002	87
(ii) + Trypsin	15 min	739	71	38645	57
	30 min	689	66	36381	48
	60 min	698	67	32840	43
(iii) No addition	15 min	878	84	63730	84
	30 min	867	83	64103	84
	60 min	812	78	61761	81

(a) Exponential cultures of LEB16 in PA medium with 20 µg/ml thymine were pulse-labelled for 10 min with either ^{14}C-D-Glutamic acid (1.8 µg/ml, 0.168 µCi/µg) or ^{14}C-L-Leucine (2 µg/ml, 0.2 µCi/µg).

thymine LEB16 exhibits balanced growth, (Fig. 8). Pulse-labelling a synchronous culture of glycerol grown LEB16 at intervals with ^{14}C D-glutamic acid (3. µg/ml; 0.168 µCi/µg) gives a similar pattern of incorporation to that described previously, (Fig. 22). Addition of chloramphenicol, a specific inhibitor of protein synthesis, during the pulse makes no alteration to the pattern of incorporation but does reduce the absolute amount of label taken up (Fig. 23). This may indicate that a proportion of the glutamate is utilised in protein synthesis. In both cases the rate of uptake of radioactive glutamate into acid-precipitable material increases with cell age. Control experiments in which the rates of protein and DNA synthesis were measured by pulse-labelling with leucine and thymidine respectively, indicate normal growth of the synchronous culture, (Fig. 24). Protein synthesis increases exponentially in rate as observed in PA medium, whereas DNA synthesis proceeds at a linear rate with a synchronous burst of initiation about 20 minutes before division.

The site of incorporation of the glutamate during growth on glycerol has been analysed by two methods. Firstly I have examined the susceptibility of the labelled material to enzyme degradations by the method described earlier (Materials and Methods, section 2.VII). Here the method has been modified by allowing digestion of the labelled material by either lysozyme or trypsin and then heating the mixtures to 100°C for 3 minutes in order to inactivate the enzyme. The mixtures were then digested with the second enzyme. Table 8 shows the results obtained

Figure 23 : INCORPORATION OF D-GLUTAMIC ACID DURING THE DIVISION CYCLE IN GLYCEROL MEDIUM

Plots of cell number (•) and radioactivity incorporated during a 5 minute pulse of ^{14}C-D-glutamic acid in the presence (Δ) and absence (■,□) of chloramphenicol (160 µg/ml), during synchronous growth of LEB16 in glycerol medium containing 20 µg/ml thymine.

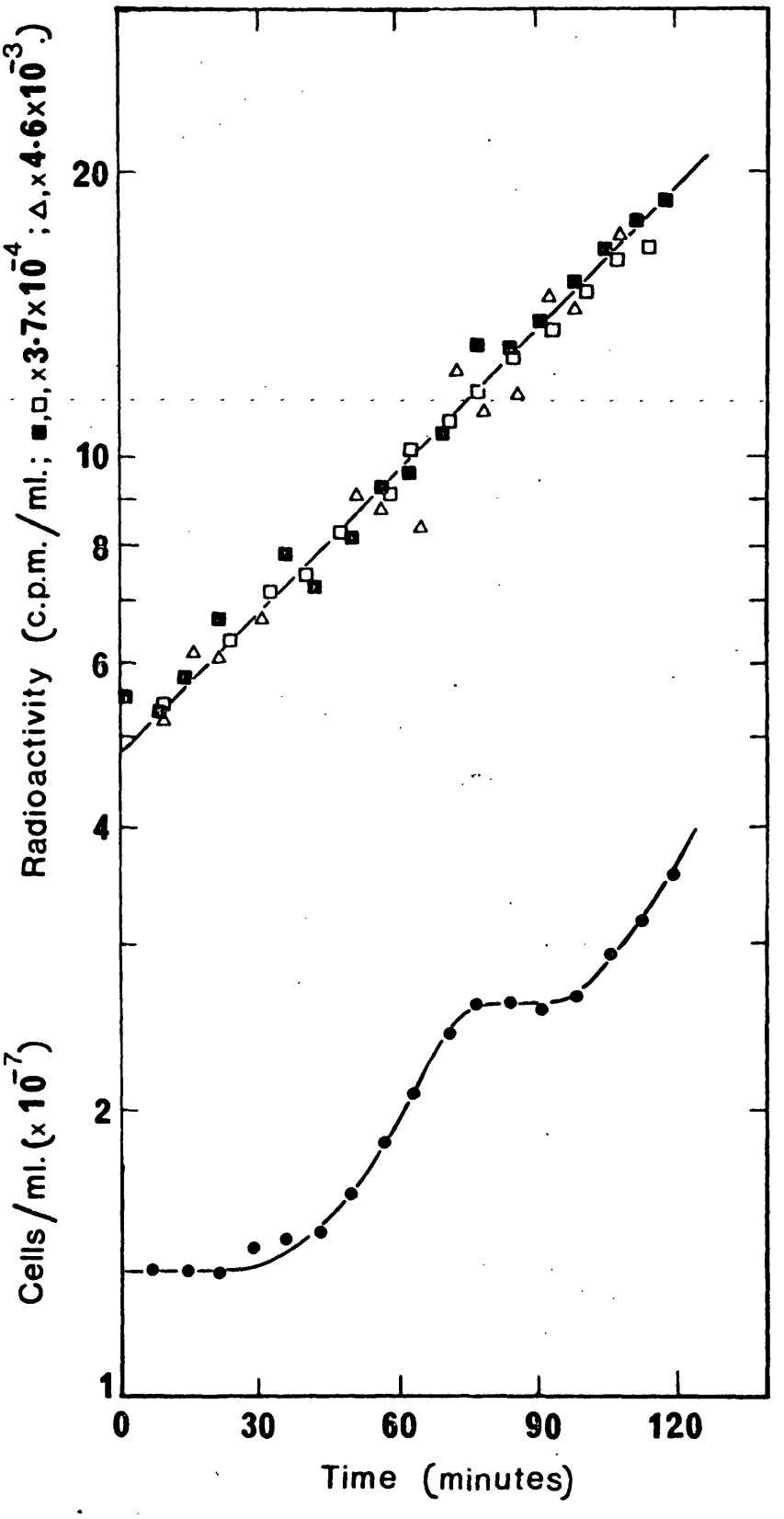

Radioactivity (c.p.m./ml.; ■,□, x3·7x10⁻⁴; △, x4·6x10⁻³)
Cells/ml. (x10⁻⁷)
20
10
8
6
4
2
1
0
30
60
90
120
Time (minutes)

Figure 24 : DNA AND PROTEIN SYNTHESIS DURING THE DIVISION CYCLE IN GLYCEROL MEDIUM

Plots of (•) cell number and radioactivity incorporated during 5 minute pulses of (□) ^{14}C-thymidine (3.2 µg/ml, 0.217 µCi/µg) and (Δ) ^{14}C-L-leucine (2 µg/ml, 0.2 µCi/µg), during synchronous growth of LEB16 in glycerol medium containing 20 µg/ml thymine.

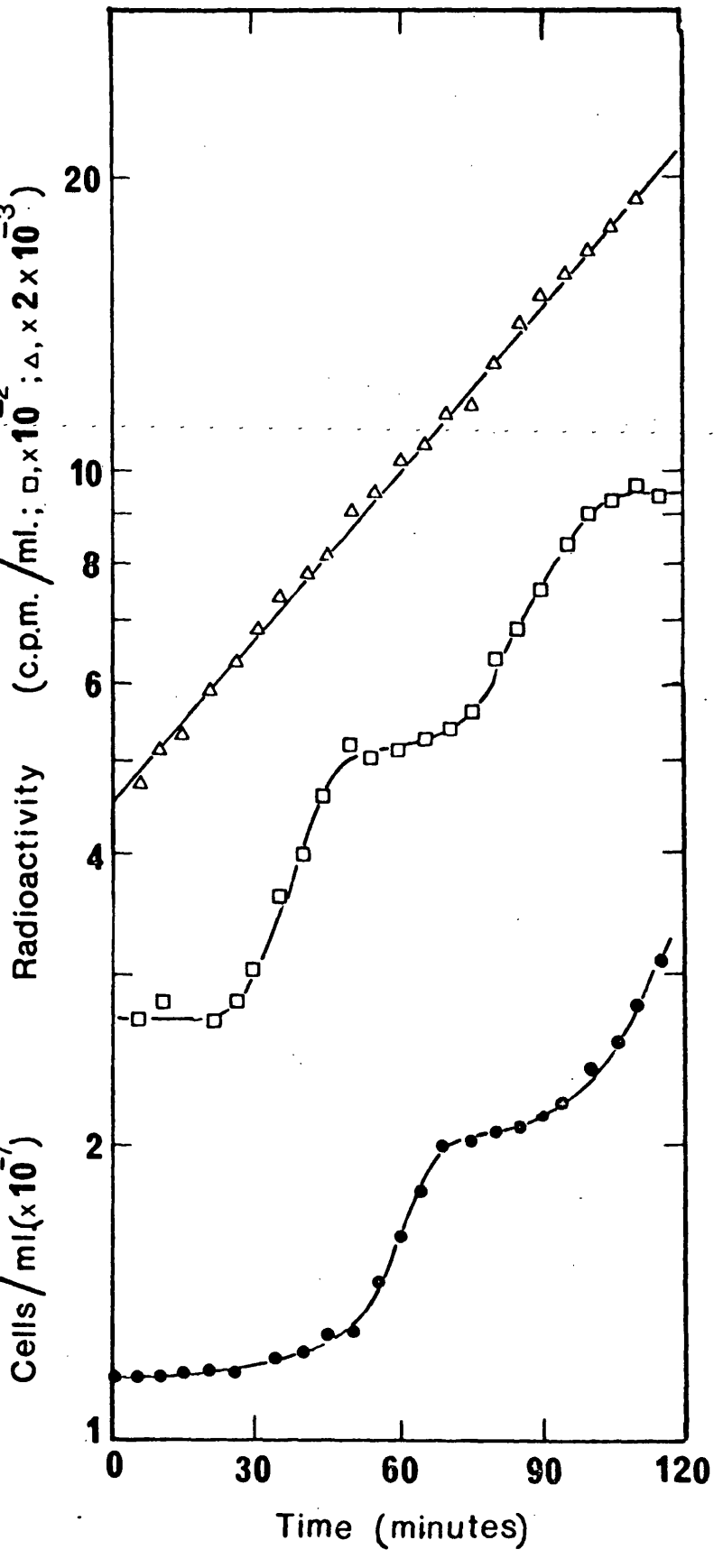

Radioactivity (c.p.m./ml.; □, x10⁻²; △, x2x10⁻³)
Cells/ml(x10⁻⁷)
20
10
8
6
4
2
1
0
30
60
90
120
Time (minutes)

Table 8. DISTRIBUTION OF RADIOACTIVITY IN CELL FRACTIONS AS DETERMINED BY ENZYME SENSITIVITY

Step		TCA precipitable activity[(a)] cts/min	%
Cell suspension		1897	100
Sonication		1814	96
100°C for 3 min		1819	96
(i) + Lysozyme	1hr	1357	72
	4hr	1382	73
100°C for 3 min + Trypsin	1hr	1096	58
(ii) + Trypsin	1hr	987	52
	4hr	809	43
100°C for 3 min + Lysozyme	1hr	442	23
(iii) No addition	1hr	1713	90
	4hr	1601	84

(a) Cells were grown in glycerol medium with 20 µg/ml thymine and labelled for 15 min with ^{14}C-D-Glutamic acid (7.45 µg/ml, 0.054 µCi/µg).

in this experiment. In glycerol grown cells only about 25% of the radioactive glutamate can be digested by lysozyme, whereas a larger amount, approximately 50% can be removed by incubation with trypsin. This substantiates the observation of the effect of chloramphenicol on the amount of incorporated material which was interpreted as indicating the use of a large proportion of the glutamate for protein synthesis.

The site of incorporation of D-glutamic acid has also been examined by a second method in which the amount of label solubilised at each stage of sequential fractionation procedure is measured. Aliquots of exponential glycerol grown cultures of LEB16 were briefly exposed to a radioactively labelled precursor, and the amount of label incorporated into various components of the acid precipitable material determined as described in Method 1, Table 1, of the Cell Fractionation procedures (Materials and Methods, section 2.VII). The results are recorded in Table 9. In the case of the leucine and thymidine labelled material the radioactivity was preferentially recovered in the "trypsin sensitive" and "hot TCA soluble" fractions respectively, as expected of protein and nucleic acid precursors. A large proportion of the radioactivity incorporated as D-glutamic acid was also found to be sensitive to trypsin digestion. However, this was not detected when chloramphenicol was present during the labelling period (Table 9). These two observations provide further evidence that the D-glutamic acid has been utilised for protein synthesis. As observed with the

Table 9. DISTRIBUTION OF RADIOACTIVITY IN CELL FRACTIONS AS DETERMINED BY SEQUENTIAL EXTRACTION

Fraction	Radio-activity extracted							
	^{14}C-D-glutamic acid[a]		^{14}C-D-glutamic acid[b] + chloramphenicol		^{14}C-L-leucine[c]		^{3}H-thymidine[d]	
	cpm/ml	%	cpm/ml	%	cpm/ml	%	cpm/ml	%
Aqueous-ethanol soluble	4090	4.8	4350	9.0	14328	3.4	604	0.3
Hot TCA soluble	35678	41.9	36226	74.7	22016	5.2	191790	97.4
Trypsin sensitive	33286	39.1	974	2.0	371498	87.8	3762	1.9
Lysozyme sensitive	10910	12.8	6666	13.8	1732	0.4	388	0.2
Residue	1113	1.3	281	0.6	13663	3.2	293	0.2

Exponential cultures (10ml) of LEB16 were grown in glycerol medium with 20 µg/ml thymine to an A_{450} of 0.25. The cultures were pulse-labelled for 10 min with (a) ^{14}C-D-glutamic acid (3 µg/ml, 0.168 µCi/µg); (b) ^{14}C-D-glutamic acid (3 µg/ml, 0.168 µCi/µg) in the presence of chloramphenicol (100 µg/ml); (c) ^{14}C-L-leucine (2 µg/ml, 0.2 µCi/µg); (d) methyl-^{3}H-thymidine (0.006 µg/ml; 77.9 µCi/µg). Incorporation was ended by the addition of an equal volume of cold 10% TCA containing unlabelled compound (100 µg/ml) as carrier. Fractionation of the TCA insoluble material was carried out as described in Method 1 of the Materials and Methods section 2.VII.

previous enzymatic digestion (Table 8) only a small proportion of the glutamate label was sensitive to lysozyme degradation. In both cases of glutamate labelled cells, a considerable proportion of the radioactivity was extractable by hot TCA treatment. The nature of this material is not known, but the treatment should solubilise only nucleic acids and other anionic polymers e.g. teichoic acids from Gram positive bacterial species. Other investigators (Park & Hancock, 1960; Roodyn & Mandel, 1960) who have used similar methods, report that in their analyses the "hot TCA" extract was contaminated with cell wall material. Since none of the nucleic acid components contain carbon atoms derived directly from glutamate it seems most probable that the radioactive label recovered in my TCA extract is also of cell wall origin. However, more extensive studies are needed to clarify this issue.

The main conclusion to be reached from these studies is that under certain conditions, namely in PA medium and possibly in glycerol medium with added chloramphenicol, D-glutamic acid is incorporated into cell wall murein. The rate of incorporation increases exponentially with cell age. Whether this reflects the true rate of murein synthesis is unresolved in these experiments. Since no indication of a "step-pattern" for murein biosynthesis could be obtained it was not possible to investigate whether control of the synthesis of this envelope component is exerted by the replication cycle.

6.III. THE EFFECT OF CHANGING REPLICATION VELOCITY ON CELL SHAPE

Reduction of the replication velocity in cultures of bacteria results in several changes to the composition of the cells in the culture. The results of preceeding sections of this thesis, and of other workers (Pritchard & Zaritsky, 1970; Zaritsky & Pritchard, 1971, 1973) have shown that a lengthening of the replication period causes an increase in average cell mass and a decrease in the DNA/mass ratio of the culture. A less obvious consequence is that the concentration of all genes, other than those at the chromosome replication origin, is reduced (Chandler, 1973; Chandler and Pritchard 1975). It therefore follows that if production of cell envelope is limited by the number of copies of a particular chromosomal locus as suggested by Pritchard (1974), a reduction in replication velocity should lead to a change in the concentration of envelope material per unit of culture mass. This change may be reflected as an alteration in cellular morphology. Since cells in balanced exponential growth increase their size by extension in length only it may be expected that any alteration in cell shape would take place in this dimension. However Zaritsky and Pritchard (1973) have reported that when thymineless bacteria undergo a reduction in replication velocity the increase in cell size is accompanied by a change in cell width rather than length. The cells appear to become more spherical thereby reducing their surface area of volume ratio i.e. the envelope/mass

ratio is reduced qualitatively as predicted.

I have made similar observation on E.coli B/r Thy⁻ LEB16. Cells from different states of balanced exponential growth were fixed with formaldehyde and examined in the electron microscope. Photographs of the preparations were used to make measurements of cell widths. Plates 1 and 2 show cells of LEB16 after growth in PA medium supplemented with high (20 µg/ml) and low (2 µg/ml) thymine respectively. In both cases the cells appear as normal rods and there is no tendency to filamentation. Measurements of cell widths (Table 10) confirms the report of Zaritsky and Pritchard that cells in low thymine are fatter than those in high thymine. As there is a distribution of cell lengths over at least a twofold range in any exponential population I have not used direct measurements to estimate changes in this dimension arising from growth in low thymine. Instead I have calculated values for average cell lengths by using the measured widths, the average cell mass data (Table 5) as an estimate of cell volume, and assuming the cells to be cylinders with hemispherical ends. All of the extra mass (volume) can be accommodated by the increase in width with the result that the cells may possibly be shorter when grown in low thymine (Table 10).

A more dramatic demonstration of this effect of growth in low thymine is provided by comparison of cells of Thy⁺ and Thy⁻ strains grown in glucose medium containing low thymine, Plates 3 & 4. In these growth conditions the Thy⁻ derivative LEB16, increases in size continuously, (Fig. 8), which has the effect of amplifying changes in

CELL SHAPE AT DIFFERENT REPLICATION VELOCITIES

Electron micrographs of E.coli B/r growing with various replication velocities. In all plates the dark spheres are latex beads (0.308 μm diameter) which are included as calibration standards.

Plate 1: LEB16 grown in PA medium supplemented with 20 μg/ml thymine.

Plate 2: LEB16 grown in PA medium supplemented with 2 μg/ml thymine.

Plate 3: LEB18 grown in glucose minimal medium.

Plate 4: LEB16 grown in glucose minimal medium supplemented with 2 μg/ml thymine.

The final magnification in all plates is approximately $X1.1 \times 10^4$.

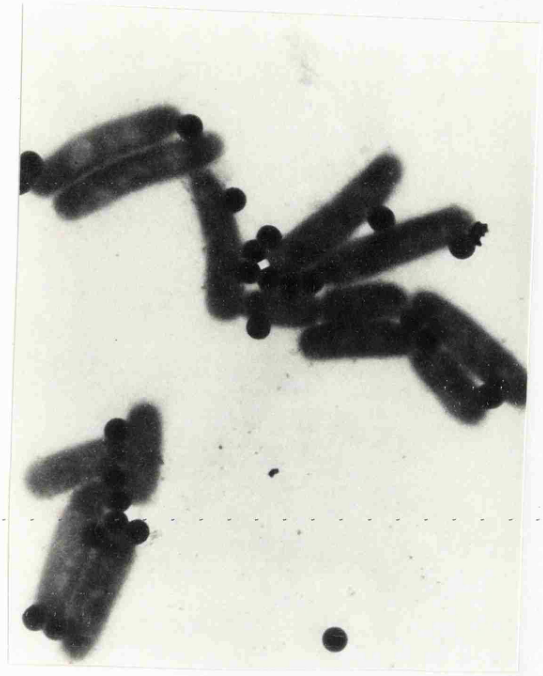


Plate 1

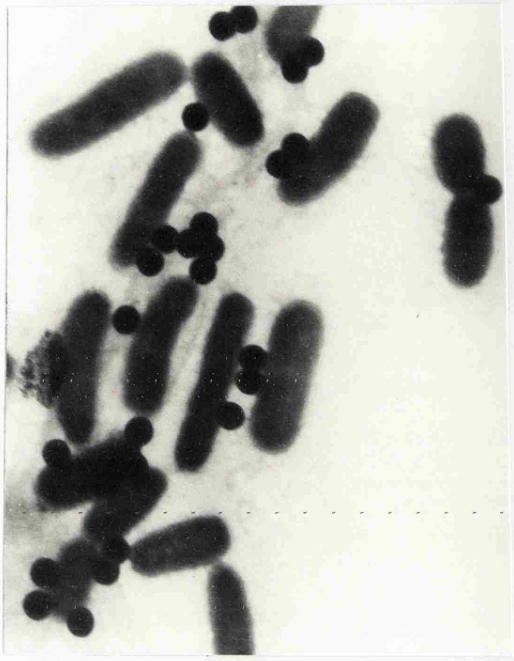


Plate 2

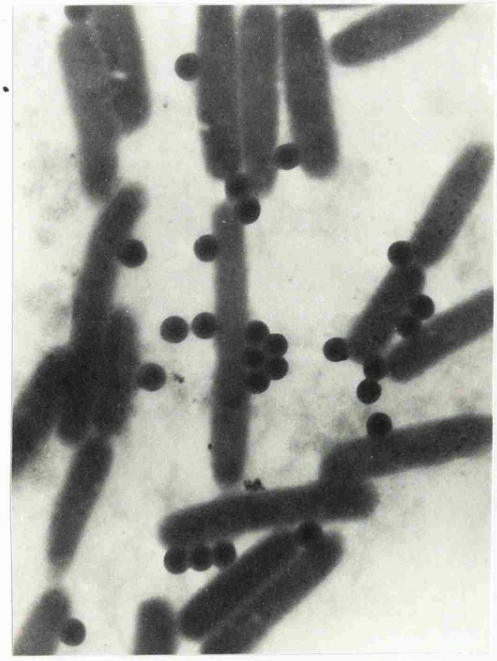


Plate 3

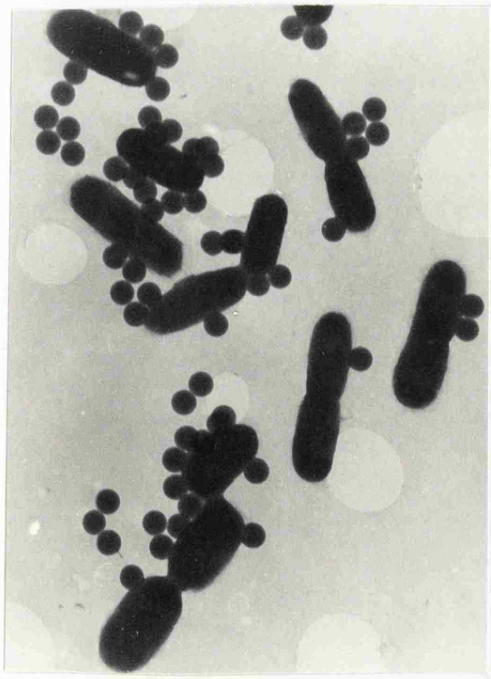


Plate 4

Table 10. DIMENSIONS OF LEB16 IN HIGH AND LOW THYMINE

Thymine (µg/ml)	Mass/Cell[a] ($A_{450}/10^9$ cells)	Mean Cell Width (µm) ± Standard Error of the Mean	Number of Cells Measured	Mean Cell Length[b]	Mean Surface[c] Area/Cell	Mean Surface[c] Area/Mass
20	1.23	0.49 ± 0.002	72	6.69	10.29	8.37
2	1.43	0.57 ± 0.004	105	5.79	10.37	7.26

(a) from Table 5

(b) Calculated using Mass/Cell as a measure of volume, and assuming cells to be cylinders with hemispherical ends

(c) Calculated from width measurements and length calculations

cell dimensions. The ratio of cell length to diameter is considerably affected in such cultures. These changes in width and length result in a reduction in the surface area/mass ratio of the cells, (Table 10).

7. GENERAL DISCUSSION

The experiments which I have described in this thesis were designed to investigate the timing of cell division in relation to the replication cycle. They were not primarily concerned with questions about which events or processes must occur before division is possible, although they may provide indirect information about this. Meaningful questions about temporal relationships are best answered by experiments in which the growth of the cells is disturbed as little as possible.

The most direct demonstration that changes in the amount of thymine supplied to thymineless mutants can cause qualitative changes to the length of the replication period C is provided by the elution experiments, (Figs. 9, 10, 12 & 13). The disappearance of the gap between rounds of DNA synthesis, with no concomitant change in doubling time, is confirmation that lowering the thymine concentration leads to an increase in transit time. That these changes cannot be quantitated accurately is a reflection on the resolution of this technique and the degree of variance in bacterial populations. In addition, it is conceivable, in view of recent observations (Werner, 1971), that the incorporation pattern using thymidine as label may be affected by repair synthesis. However Helmstetter (1974) has shown that the incorporation pattern of thymidine during the division cycle of E.coli B/r is essentially the same as the pattern of incorporation of thymine by a thymineless mutant. Furthermore the DNA/mass ratio measurements, which were made

using thymine instead of thymidine do not experience this source of error and provide independent evidence for a lengthening replication period, (Table 4). Quantitative estimates of the changes induced in the cell cycle parameters C and D by thymine limitation can be obtained from measurements of this type on steady-state exponential cultures, (Tables 4 and 5; Pritchard & Zaritsky, 1970; Zaritsky & Pritchard, 1973). If the assumptions (Pritchard & Zaritsky, 1970; Zaritsky & Pritchard, 1973) underlying the equations used for these calculations are correct for the conditions under which the measurements are made, then the estimates of C and D that are obtained will not be in error. The validity of these assumptions is further corroborated by the correlation between the observed patterns of DNA synthesis as a function of cell age, and those predicted from the calculated values of C and D, (Fig. 14). All of the data are compatible with the view that initiation of chromosome replication occurs at a constant mass per chromosome origin (Donachie, 1968), which is unaffected by variation in thymine concentration (Chandler & Pritchard, 1975; Pritchard & Zaritsky, 1970).

The results of experiments by two independent methods show too that increases in the length of the replication time C are accompanied by decreases in the period D between termination and division, although these changes may not be exactly complementary, (Table 5). Thus the length of the D period is not invariant as originally suggested (Cooper & Helmstetter, 1968). It is different in cultures with different transit times even though they have identical

growth rates. The same conclusion can be reached from the data of Zaritsky and Pritchard (1973) for E.coli 15T⁻. Only by assuming a reduction in D can the changes in average cell mass be reconciled with the increments in C calculated from the DNA/mass ratio measurements. These results are clearly incompatible with the model whereby division is timed from initiation and D is the time difference between the replication and division processes (Jones & Donachie, 1973; Shannon & Rowbury, 1972). In this case complementary changes in C and D are predicted. Furthermore, there should be no increase in average cell size ($\bar{M}$) until C exceeds C+D. The steady-state analysis (Table 4) indicates that cell size, and therefore C+D, increases even for small increments in the transit time.

The results of the "step-up" and "step-down" experiments. (Figs. 16 & 17), in which the culture moves from one steady-state of growth to another, lead convincingly to the conclusion that the time of cell division is determined by a late event in the replication cycle. Moreover the time-determining event occurs at different cell ages in different steady-state conditions. In the "step-up" transition the period between the "timing" of division and the division process per se is very short, whereas in the "step-down" situation the delay is of the order of 20 minutes. The fact that this difference closely corresponds to the period between termination and cell division (D) in the pre-step conditions, as determined from the elution data (Figs. 12 & 13), and steady-state analysis (Table 5), supports the hypothesis that termination

provides a signal which determines the time at which the ensuing division will occur. To this extent the data are in agreement with the original hypothesis (Clark, 1968a,b; Helmstetter et al. 1968) and inconsistent with the alternative suggested by others (Jones & Donachie, 1973; Shannon & Rowbury, 1972).

Termination as a pre-condition for division is evidenced by the measurements of the extent of residual division of exponential cultures after addition of the DNA synthesis inhibitor nalidixic acid, (Fig. 19). Since the size of the increment in different growth states is equivalent to the proportion of the cell population which is in the D period (Table 6), the data support the conclusion that only those cells which have completed a round of replication are able to complete division. All of the data discussed here are consistent with the view that there is a one to one relationship between termination and division, although the time interval between the two may vary. The notion of a fixed D period (Cooper & Helmstetter, 1968) is too simple.

To try to understand why the time from termination to division does change in this way it is necessary to ask how changes in C influence the cell. It is perhaps significant in this context that when C is lengthened the extra cell mass is taken up by an increase in cell width (Table 10 and Zaritsky & Pritchard, 1973), thereby reducing the surface area/volume ratio. Such an increase in diameter has one obvious consequence it will increase the amount of envelope material needed to construct the septum.

This can hardly lead to the difference in D that is observed since it is the larger cells that have the shorter D, (Table 5). A less obvious effect of an increase in cell diameter is that it causes a larger increment in cell volume for any given addition to the cell's length. This may be a significant factor leading to the changes observed in D.

If as some evidence (Kubitschek, 1968; Kubitschek, 1970; Ward & Glaser, 1971) suggests, cells increase in volume at a linear rate with doublings in this rate occurring once every cell cycle, and if cell mass increases exponentially (Ecker & Kokaisl, 1969), then the mass/volume ratio will vary continually during the cell cycle. It has been suggested (Pritchard, 1974) that this could lead to a corresponding oscillation in the internal hydrostatic pressure, and it is only at the lower end of this oscillatory variation, which will occur shortly after the doubling in rate, that the cell is able to devote its envelope synthesising capacity to septum formation instead of extension in length.

If, under steady-state conditions, cells of E.coli increase their volume by extension in length only (Marr et al. 1966) then a specific prediction of any model of this type is a "stepwise" increase in the rate of surface growth associated with a particular stage of the replication cycle. Pulse-labelling studies using radioactive D-glutamic acid to measure the rate of synthesis of cell wall murein as a function of cell age do not support this model, but

show an exponentially increasing rate of synthesis during the division cycle, (Figs. 21 & 22). A similar conclusion is reached from the data of Ryter *et al* (1973). In an autoradiographic study of sacculi which had been briefly allowed to incorporate radioactive diaminopimelic acid, they observed that the average grain count per cell, a measure of the rate of incorporation, increased with cell size in both fast and slow growing cells. In contrast Hoffmann *et al*. (1972) report that the rate of D-glutamate incorporation does increase in a stepwise manner in glucose grown synchronous cultures of *E.coli B/r*. There is no immediate explanation for the discrepancy between these results. Although under certain conditions a large proportion of the D-glutamate incorporated in my experiments appear to be in murein (Tables 7 & 9), it is possible, because of the uncertainties regarding D-glutamate biosynthesis (Lugtenberg *et al*. 1973), that the observed rate may not be a true estimate of the synthesis of murein but may be affected by endogenously supplied material. This question can only be resolved by more extensive biochemical analysis into the fate of the incorporated glutamate. On the other hand the exponential increase may be a true reflection of the rate of murein synthesis. If so the increasing rate may be due to the continuous introduction of new synthesis sites as the sacculus is enlarged to parallel the increase in cell mass. From studies on the cell wall composition of normal and morphologically mutant *E.coli* cells it has been suggested

(Henning, Rehn, Braun & Hohn, 1972; Henning, Rehn & Hoehn, 1973; Schwarz & Leutgeb, 1971) that the murein layer is unable to actively adopt a specific conformation but is passively synthesised around some other morphogenetic structure. In such a case an analysis of the rate of synthesis of the various components of the shape-determining structure may provide more direct information concerning the nature of any regulation on envelope synthesis mediated by the replication cycle.

That there is a control on envelope synthesis by the replication cycle is, however, apparent from the measurements of the dimensions of cells growing with different replication velocities. As the doubling time remains unaltered under these conditions the decreasing surface area/mass ratio (Table 10) implies that a lengthening of the C period causes a reduction in the differential rate of envelope synthesis. A relationship between termination of a round of replication and the synthesis of cell envelope has been postulated (Zaritsky & Pritchard, 1973; Pritchard, 1970) to account for this finding, the suggestion being that there is either an identity between the number of cell elongation sites and chromosome termini or a direct dependence of the rate of envelope synthesis on the output of an unregulated gene located at the terminus. Analysis of the dimensions of S.typhimurium growing at different rates favours the former of these two possibilities (Zaritsky & Pritchard, 1973). However since it is

necessary to assume some sort of proportionality between the rate of envelope production per site, or gene, and the growth rate, neither alternative can be unambiguously excluded (Pritchard, 1974).

The shape changes arising as a result of replication velocity changes should permit a distinction to be made since there is no alteration in growth rate and therefore no alteration in the degree of non-specific control of gene expression e.g. catabolite repression. If the number of elongation sites is determined by chromosome termini then average cell length should vary in proportion to changes in D. By use of the age distribution theorem (equation (1), Powell, 1956) it can be shown that the average number of termini per cell, $\bar{T}$, is given by (Bleecken, 1969; Kubitschek, 1974):

$$\bar{T} = 2^{D/\tau} \quad \ldots\ldots\ldots\ldots (7)$$

Similarly if total envelope production is dependent upon the output of a terminal gene then the surface area/mass ratio should change in proportion to the concentration of the gene. Chandler (1973; Chandler & Pritchard, 1975; see also Pritchard, 1974) has shown that the concentration, $\bar{F}/\bar{M}$, i.e. the number of copies per unit mass, of a gene located at a fractional distance x from the replication origin is given by

$$\bar{F}/\bar{M} = \frac{1}{k.}\ 2^{-Cx/\tau} \quad \ldots\ldots\ldots\ldots (8)$$

For a terminal gene x is equal to 1 and thus the change in concentration becomes a function of $2^{\Delta C/\tau}$, where ΔC is the increment in C.

Table 11 shows a comparison between the predicted changes in these parameters, based upon the values of C and D calculated earlier, and the measured changes in the dimensions of LEB16. The closest correlation is found between the ratio of average cell lengths and the change in the number of termini per cell $2^{\Delta D/\tau}$, where ΔD is the decrease in D. The data thus substantiate the claim (Zaritsky & Pritchard, 1973) that the rate of linear extension is proportional to the number of termini per cell. The change in the surface area/mass ratio (0.86) is considerably less than that predicted for the concentration of termini (0.73). This can only be attributed to this particular increment in C, 27 minutes, if it is assumed that the "rate-limiting" gene is located at a fractional distance x = 0.56 on the replication map.

Zaritsky (1975, and personal communication) has pointed out that an implication of a proportionality between average cell length and the number of chromosome termini, is that changes in cell width are proportional to changes in C, as given by $2^{\frac{C}{2\tau}}$. This conclusion is reached by assuming firstly that the cells have the shape of regular cylinders where volume is given by $\pi r^2 l$, and secondly that average cell mass $\bar{M}$ (see equation 3) is proportional to volume. It follows from this that, to a first approximation, the change in average surface area per cell will be given by $2^{(C/2 + D)/\tau}$. These conclusions are also supported by the data in Table 11.

Table 11. COMPARISON OF OBSERVED AND PREDICTED CHANGES IN CELL DIMENSIONS

Observed				Change (Ratio $^{2}/_{20}$)
Thymine (μg/ml)		20	2	-
Mean Cell Width (μm)	(a)	0.49	0.57	1.16
Mean Cell Length	(a)	6.69	5.79	0.86
Mean Surface Area/Mass	(a)	8.37	7.26	0.86
Predicted				
C (min)	(b)	41	68	-
D (min)	(b)	22	9	-
Termini/Cell ($2^{D/\tau}$)		1.26	1.10	0.87
Termini/Mass ($2^{-C/\tau}$)		0.65	0.48	0.73
Theoretical Cell Width ($2^{C/2\tau}$)		1.24	1.44	1.16

(a) from Table 10

(b) from Table 5

Thus it appears from these results that the length of a bacterial cell is determined by the number of chromosome termini and that changes in cell width caused by transition between different steady-states of growth are due to the increase in internal pressure resulting from the reduction in the differential rate of volume increase. These cell shape changes do not in themselves, provide an explanation of the relationship between C and D. They are, however, consistent with predictions, using independently calculated values of C and D, of a model wherein the rate of surface growth is coupled to the replication cycle. It is possible then, that the real explanation of the D period may be found within the framework of a linear-log relationship between cell surface and mass growth.

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SUMMARY

The relationship between chromosome replication and cell division has been investigated in a thymineless mutant of E.coli B/r. The investigation is based upon the ability to vary the rate of DNA chain elongation by manipulation of the thymine concentration in the growth medium.

Examination of the changes in average cell mass and DNA content of exponential cultures arising from a reduction in replication velocity suggest that the resulting increase in the replication time C is accompanied by a decrease in the period, D, between termination of the round of replication and the subsequent cell division. Observations on the pattern of DNA synthesis during the division cycle are consistent with this relationship. Nevertheless, the kinetics of transition of exponential cultures moving between steady-states of growth with differing replication velocities provides evidence to support the view that the time of cell division is determined by termination of rounds of replication under steady-state conditions.

Measurements taken from electron micrographs of cells growing with differing replication velocities show that the increase in average cell mass is accommodated by an increase in cell width rather than length. The morphological changes are consistent with the predictions of a model for cell growth whereby the number of sites for

cell elongation, which operate at a constant rate, are equivalent to the number of chromosome termini per cell. Preliminary studies on cell wall murein synthesis indicate an increasing rate with cell age.

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Relationship Between Chromosome Replication and Cell Division in a Thymineless Mutant of *Escherichia coli* B/r

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The relationship between chromosome replication and cell division was investigated in a thymineless mutant of *Escherichia coli* B/r. Examination of the changes in average cell mass and DNA content of exponential cultures resulting from changes in the thymine concentration in the growth medium suggested that as the replication time (C) is increased there is a decrease in the period between termination of a round of replication and the subsequent cell division (D). Observations on the pattern of DNA synthesis during the division cycle were consistent with this relationship. Nevertheless, the kinetics of transition of exponential cultures moving between steady states of growth with differing replication velocities provided evidence to support the view that the time of cell division is determined by termination of rounds of replication under steady-state conditions.

Studies by Helmstetter and Cooper (5, 9) on the rate of DNA synthesis during the cell cycle of *Escherichia coli* B/r have shown that over a wide range of growth rates the period between initiation of a round of chromosome replication and the resulting cell division remains constant. Their observations not only confirm previous suggestions (see reference 15) that the time (C) taken to replicate the chromosome is constant, and therefore independent of the growth rate, but also demonstrate that there is another constant period (D) between termination of a round of replication and cell division. Thus division occurs $C + D$ minutes after each initiation event. To explain these observations it has been suggested that the control of cell division is linked to an event in the replication cycle.

Investigations into the effects of an inhibition of DNA synthesis on cell division, in both exponential and synchronous cultures, have led Helmstetter and Pierucci (11) and Clark (2) to propose that termination of a round of replication provides a signal for cell division which occurs at a fixed time, D minutes later. Evidence supporting this view has been forwarded by Hoffman et al. (13) from their finding that the rate of murein synthesis increases at a late stage in the division cycle. These authors have postulated that termination is a signal for polarcap formation, and D therefore represents the time necessary for synthesis and assembly of the cross wall.

On the other hand, it has been argued that since division can continue in the absence of DNA synthesis when temperature-sensitive initiation defective mutants of *Escherichia coli* and *Salmonella typhimurium* are shifted to the nonpermissive temperature (12, 20), processes leading to division might be triggered earlier in the cell cycle, perhaps at the time of initiation and proceed independently of on-going replication (14, 19). Although it is also assumed by these authors that termination of a round of replication is a requirement for cell division, it is argued that it does not determine the time at which it occurs: D merely represents the time difference between completion of these "division-processes" ($C + D$) and termination of the round of replication which was initiated at the same time.

One method by which these two models might be distinguished has been outlined by Zaritsky and Pritchard (22). It exploits their discovery that the replication velocity can be varied independently of the growth rate in cultures of thymineless bacteria by adjustment of the thymine concentration in the growth medium (18). If, as these authors have demonstrated, under such conditions the replication time C can be extended, then an analysis of any accompanying changes in the D period will differentiate between the two models. For although both explain the constant time interval ($C + D$) between initiation and division, they lead to different predictions about the length of D under conditions of changing C. If termination sets into operation a sequence of events resulting in cell division, then D should remain

constant and independent of changes in C, whereas if initiation provides the signal an inverse relationship between C and D is predicted so that as C is increased D will decrease.

We report here a series of experiments based upon this approach to the problem. Using a thymine-requiring mutant of *E. coli* B/r we have found, both from measurements on age-fractionated cultures and from observations of cell sizes and compositions in exponential cultures, that an increase in the length of C is accompanied by a decrease in D. This is clearly in contradiction to the first hypothesis. In addition we have examined the kinetics of the transient change in the rate of cell division which is found when exponential cultures are transferred from one thymine concentration to another. These experiments show that the timing of cell division is determined by a late event in the replication cycle in contradiction to the predictions of the second hypothesis. Thus neither model provides a satisfactory description of the cell cycle of *E. coli* B/r.

(This work was carried out in partial fulfilment of the Ph.D. requirement of P. M. at the University of Leicester, Leicester, England.)

MATERIALS AND METHODS

Bacteria. The strain used in this investigation, LEB16, is an F⁻ *lacZ, str* segregant of an F′ lac⁺ *E. coli* B/r obtained from M. Pato. It carried chromosomal mutations at *thyA* and *drm*, thereby enabling it to grow in the presence of low concentrations of thymine, i.e., less than 20 μg/ml. The *thyA* mutation is less than 5% leaky as measured by [^{14}C]uracil incorporation in the absence of exogenous thymine (P. Meacock, unpublished observations). LEB18 is a spontaneous *THY*⁺ derivative of LEB16.

Media. The minimal salts medium used was that described by Helmstetter (7) to which L-proline and L-alanine were added as carbon source, at concentrations of 0.4% (PA medium). Thymine, the only variable, was added at the concentrations indicated. This growth medium routinely gave generation times of 65 min at 37 C.

Membrane elution procedure. The experimental technique was essentially that of Helmstetter (7). Exponential cultures (100 ml), at a cell density of approximately 10^8/ml, and containing thymine at the required concentration, were pulsed for 3 min with [2-^{14}C]thymidine (1 μg/ml, 0.25 μCi/μg). The pulse was ended by binding the cells to the surface of a membrane filter (Millipore, grade GS, 0.22-μm pore size). The cells were washed with 100 ml of minimal medium, and the apparatus was inverted for elution of the dividing cells with prewarmed growth medium. Elution rate was rapid (22 ml/min) for the first 6 min to remove the majority of "unbound" cells and then reduced (6 ml/min) for the remainder of the elution period. Samples were collected continuously throughout the experiment. Cell density in the eluate was determined with an electronic particle counter (Coulter, model B, fitted with a 30-μm orifice) on samples diluted into 0.9% saline containing 0.8% formaldehyde. Radioactivity in the eluted cells was measured by precipitating 10-ml samples of the eluate with trichloroacetic acid (final concentration 5%) and holding at 0 C for 30 min; the samples were then collected by suction on 27-mm membrane filters (Sartorius, 0.45-μm pore size), washed six times with 20 ml of 95 C distilled water, and dried under an infrared lamp. The filters were placed with a constant orientation in small vials which were filled with scintillation fluid (17), stoppered, and placed in turn into standard Packard vials. The samples were counted in a Packard scintillation counter.

Measurement of average cell size and DNA composition in steady-state exponential cultures. The growth medium was inoculated with bacteria from a minimal agar plate and incubated with vigorous aeration at 37 C in a New Brunswick gyratory shaker. Culture mass was measured as absorbance at 450 nm by means of a Gilford microsample spectrophotometer. Particle number was determined by use of the Coulter counter on samples taken into ice-cold saline (0.9%) with formaldehyde (0.4% final concentration). Relative DNA contents were measured by the incorporation of [2-^{14}C]thymine (0.05 μCi/μg) from the growth medium into trichloroacetic acid-precipitable material. Samples were taken over several generations of growth and only when the culture was in a steady state of exponential growth (1, 18). For measurements of steady-state composition the absorbance at 450 nm (A_{450}) was kept in the range 0.05 to 0.2 by dilution with fresh prewarmed growth medium after each doubling in absorbance. Changes of thymine concentration during the course of an experiment were achieved by dilution of the culture with medium containing thymine sufficient to give the required final concentration.

RESULTS

Pattern of DNA replication during the division cycle as a function of thymine concentration. The patterns of chromosome replication during the division cycle of LEB16 growing in various concentrations of thymine were determined by the membrane elution technique. For each experiment an exponential steady-state culture, in a prescribed thymine concentration, was briefly exposed to radioactive thymidine, and the progeny cells were fractionated according to their ages so that their radioactive content could be measured.

The method of interpretation of this type of experiment has been fully discussed elsewhere and will therefore not be described here (see, for instance, references 4, 7, and 23).

Figure 1 shows the results of a series of experiments using LEB16 growing in PA me-

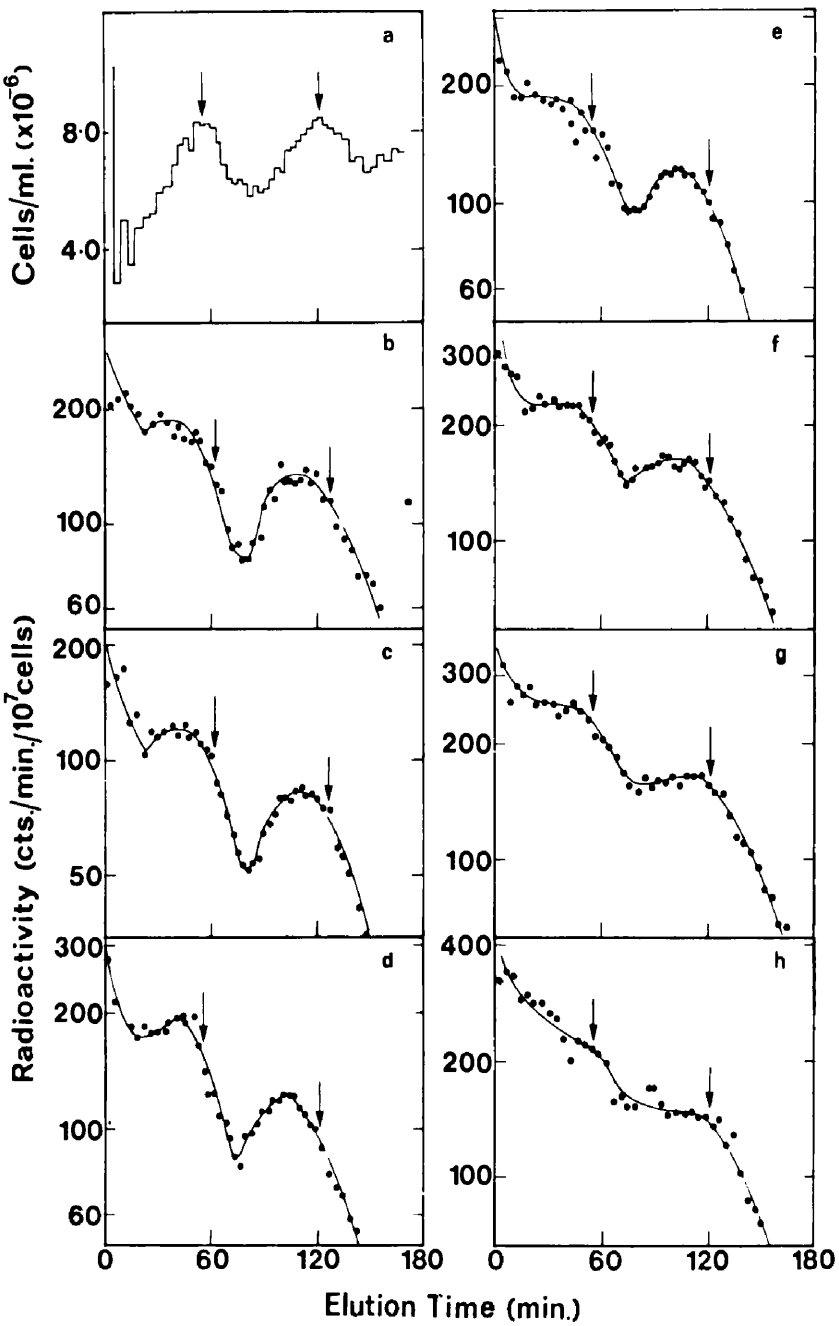

FIG. 1. *Elution patterns of cultures grown in different thymine concentrations. Plots of a typical profile of effluent cell density (a) and radioactivity per effluent cell (b through h) from [^{14}C]thymidine pulse-labeled membrane-bound populations. (b) E. coli B/r Thy$^+$, LEB18; (c, d, e, f, g, h), E. coli B/r Thy$^-$ (tlr), LEB16 grown in media containing 20, 5, 4, 3, 2, 1.5 μg of thymine, respectively. The arrows denote the positions of the peaks of the cell density profile.*

dium with thymine concentrations ranging from 20 to 1.5 μg/ml. Balanced exponential growth over this range of thymine concentrations is given by this medium (see below). Also included is the result of a similar experiment on a *THY*$^+$ revertant, LEB18, and a typical profile of the eluted newborn cells. The shape of this elution profile is unaffected by the amount of thymine in the medium (data not shown). This indicates that in all experiments the cells are in similar states (of balanced exponential growth). In our system we found that the unbound cells take approximately 10 to 15 min to be completely removed from the membrane, thus tending to obscure the early part of the elution curves. However, once these cells have been removed the elution gives a reproducible oscillatory pattern as shown in the top left-hand frame of Fig. 1.

A clear picture of the pattern of deoxyribonucleic acid (DNA) replication can be obtained from the second cycle of the elution curve. Here

it can be seen that in both THY^- cells growing in high thymine (20 μg/ml) and in THY^+ cells the replication cycle is essentially the same and agrees well with previous estimates for C and D of 41 and 22 min, respectively (5). However, as the thymine concentration is lowered the pattern of replication changes, the most significant difference being the disappearance of the gap in DNA synthesis, as indicated by the absence of the trough in the elution curve at 70 to 80 min, on the 2 μg/ml data.

Thus the pattern of replication in low thymine is similar to that observed for wild-type cells growing in glucose minimal salts medium (3, 8). Completion of the round of replication is no longer detected as a decrease in the rate of incorporation because it is obscured by the succeeding initiation event which now occurs at the same time. Since the spread of the elution profile is not greatly affected by the different thymine concentrations, we conclude that the main change resulting from a reduction in the thymine concentration is a lengthening of the DNA synthesis period.

If D remains constant, then a progressive lengthening of C should lead to a corresponding progressive movement of the initiation event to an earlier cell age. Alternatively, an increase in C at the expense of D would result in no change in the age at which initiation occurs, but would cause a movement of termination to a later cell age. Both possibilities result in the same eventual qualitative effect, namely, the coincidence of initiation and termination. In Fig. 2 we have replotted the data from the second generation of the elution curves on a cell age basis. Although we have taken the peak-to-peak cycle for this plot, and a more accurate description might have been obtained from the half-height cycle (see reference 10), this is unimportant in this content since it is the overall pattern of changes that we are concerned with.

It is difficult to draw definite conclusions about the nature of the relationship between C and D from these data. Although initiation remains late in the cell cycle, the data of Fig. 2 appears to show that it may be occurring somewhat earlier in the low thymine cases. However, this change is probably not sufficient to account for the complete disappearance of the gap between rounds of replication. It is therefore possible that an accompanying displacement of termination towards division and towards initiation also takes place. The changes are not sufficiently large to permit a clear distinction of the mode of change of C and D.

Another point which should be noted from Fig. 1 is that although the levels of radioactivity per cell cannot be directly compared because of the technical difficulties in exactly controlling the pulse time and conditions, it does seem that the number of replication forks per cell is not greatly different in high and low thymine. Therefore the changes in the length of C are not so large as to result in extensive periods of dichotomous replication.

Measurement of cell size and DNA composition as a function of thymine concentration. We have seen from the membrane elution experiments described above that variations in the exogenous thymine concentration cause qualitative changes in the pattern of DNA replication during the cell cycle of LEB16. Since there is a considerable spread around the mean of the cell ages at which events such as termination and initiation are occurring, as indicated by the general roundness of the elution curves, we feel that it is difficult to obtain accurate estimates of the changes in C and D from this type of experiment. It therefore seems prudent that we should use a second unrelated method which does not experience this potential source of error and provides an independent estimate of the effect of changes in C on D. A quantitative assessment of changes in these parameters can be made from measurements of the size and DNA content of cells in exponential steady states of growth in different thymine concentrations.

Previous investigators (6, 9, 16, 18) have shown that when a culture is in a balanced state of exponential growth the DNA content ($\bar{G}$) and mass ($\bar{M}$) of the average cell and the DNA-mass ratio ($\bar{G}/\bar{M}$) of the culture can be described as functions of the three cell cycle parameters C, D and τ.

$$(\bar{G}) = \frac{\tau}{C.\ln 2}\left(2^{(C+D)/\tau} - 2^{D/\tau}\right) \qquad \text{(i)}$$

$$(M) = k.2^{(C+D)/\tau} \qquad \text{(ii)}$$

$$(\bar{G}/\bar{M}) = \frac{\tau}{k.C.\ln 2}\left(1 - 2^{-C/\tau}\right) \qquad \text{(iii)}$$

If k, the initiation mass (6), is unaffected by variations in C, (18, 22; M. Chandler and R. H. Pritchard, Mol. Gen. Genet., in press), then changes in C will lead to corresponding changes in $\bar{G}$, $\bar{M}$, and $\bar{G}/\bar{M}$. The magnitude of these changes will, in the cases of $\bar{G}$ and $\bar{M}$ but not of $\bar{G}/\bar{M}$, depend upon the value of D for each increase in C. Therefore, by comparing variations in these three parameters, with predictions calculated from the equations for different C values, it should be possible to deduce whether

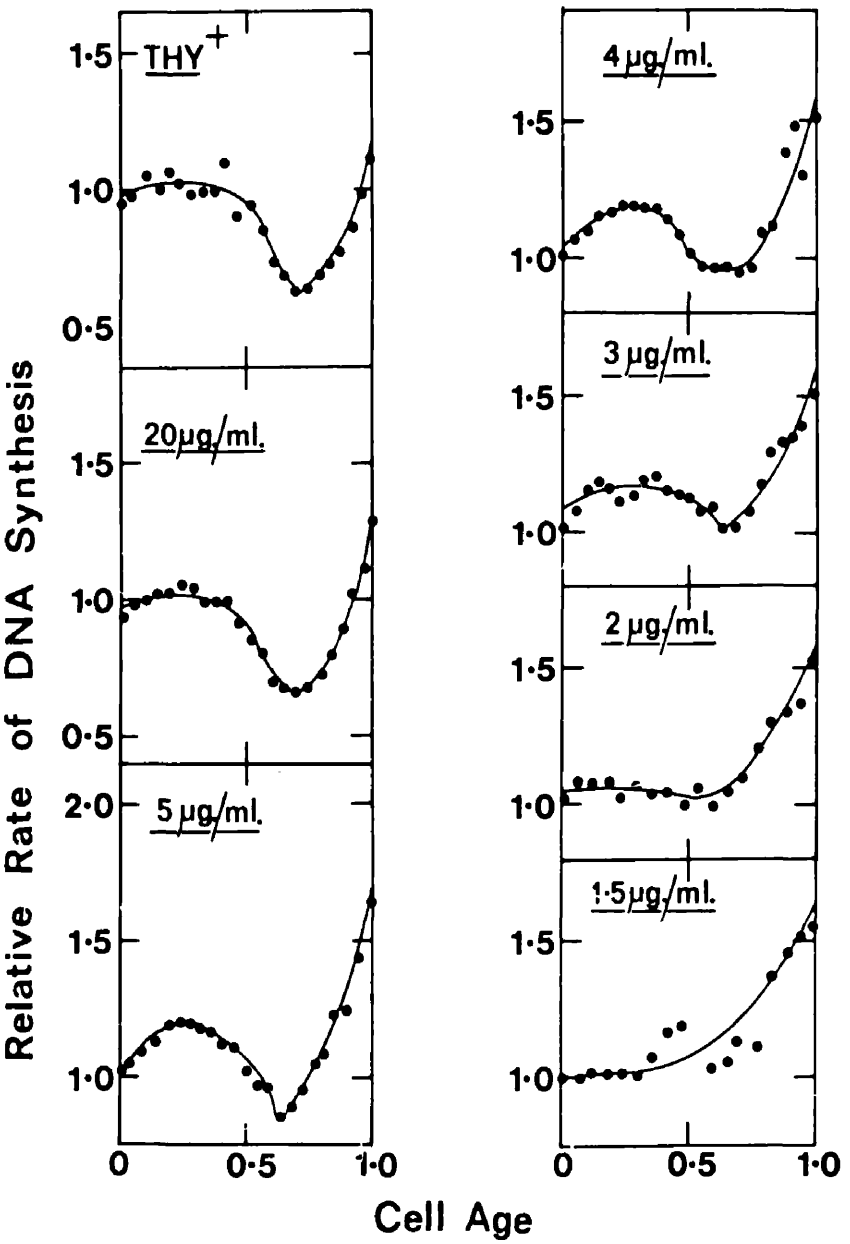

FIG. 2. *Relative rate of DNA synthesis over the cell cycle at different thymine concentrations. The figure is a replot of the curves (b through h) in the second generation of elution in Fig. 1, i.e., the position of the elution curves between the two arrows. The data have been normalized to an initial rate of DNA synthesis of 1.*

there is any alteration to the length of the *D* period.

It is important when making such measurements to ensure that the culture is at all times in a balanced steady-state of exponential growth (1, 18), as indicated by the parallel increase of all measured quantities. Under the conditions of the experiments discussed here LEB16 does show balanced growth. In Fig. 3a and b it can be seen that when this strain is grown in the PA medium with concentrations of thymine of 20 or 1.5 μg/ml, absorbance, particle number, and DNA content all increase at the same rate. Also shown are measurements taken on a culture growing in 1.0 μg of thymine per ml (Fig. 3c); here the culture is once again in exponential growth, but the growth is not balanced. Although absorbance and DNA content increase together, cell number increases at a slower rate. In this situation average cell mass $\overline{M}$ continues to increase, whereas the DNA-mass ratio $G/\overline{M}$ remains constant (Fig. 4). We emphasise that in all cases the doubling time of the cultures as measured by A_{450} are identical; only at even lower thymine concentrations (0.5 μg/ml) does the growth rate become reduced (P. Meacock, unpublished observations). PA medium was used because in media allowing a faster growth rate the apparent uncoupling between cell growth and division occurs at

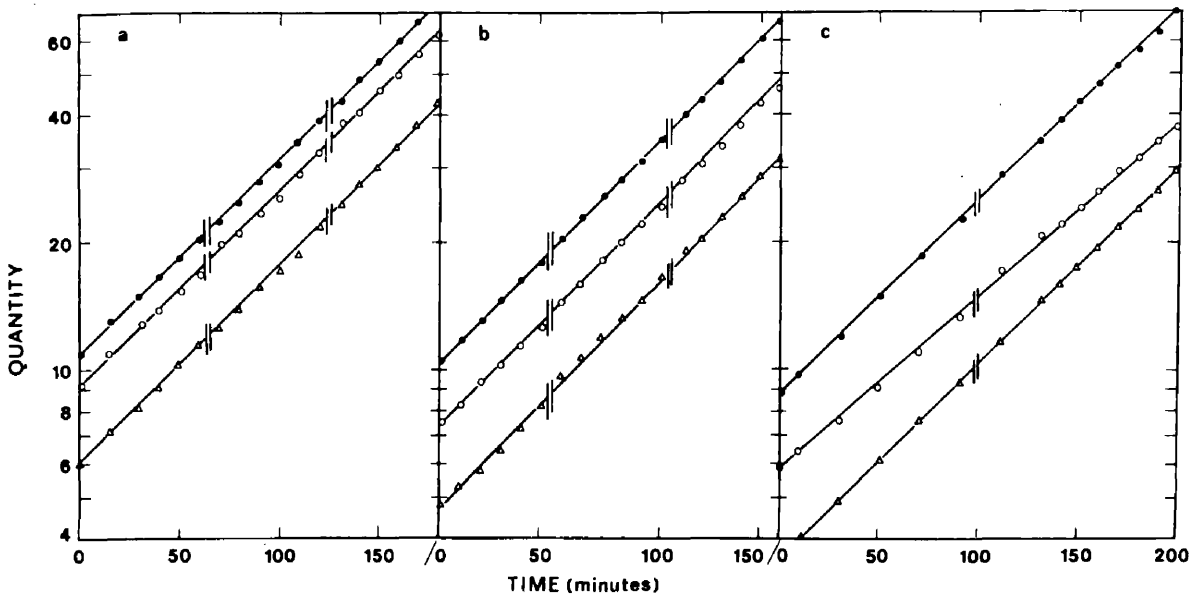

FIG. 3. *Effect of thymine concentration on the rate of increase in absorbance, cell number, and DNA in LEB16 grown in PA medium. A_{450} (10^{-2}) (●), cell number (10^7/ml) (○), and counts per minute (10^3/ml) (△) in cultures uniformly labeled with [^{14}C]thymine (0.05 μCi/μg) at concentrations of (a) 20 μg/ml, (b) 1.5 μg/ml of and (c) 1.0 μg/ml. After each doubling in cell number the cultures were diluted twofold with fresh prewarmed medium. The points plotted are corrected for these dilutions.*

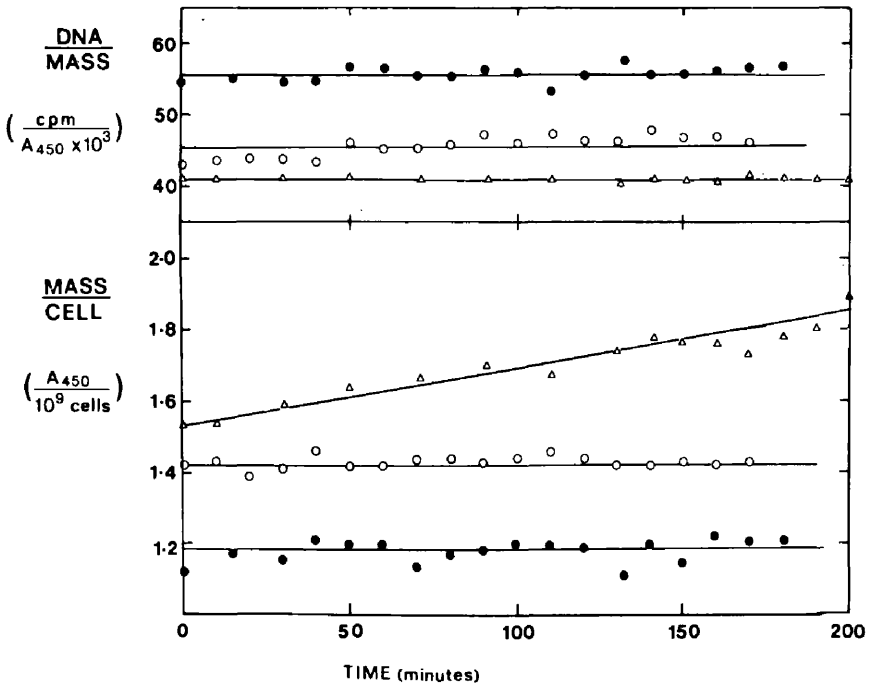

FIG. 4. *DNA-mass ratio ($\bar{G}/\bar{M}$) and average cell mass ($\bar{M}$) for LEB16 grown in PA medium with thymine concentrations of 20 μg/ml (●), 1.5 μg/ml (○), and 1.0 μg/ml (△). The values are calculated from the data in Fig. 3.*

higher thymine concentrations. Thus in glycerol medium growth is unbalanced in 10 μg of thymine per ml, and the degree of unbalance is even greater in glucose medium at this concentration (Fig. 5). In another thymineless mutant (*E. coli* 15T$^-$) (18, 22) it has similarly been shown that the minimum concentration of thymine required for balanced growth increases with growth rate.

Table 1 shows the results of a series of experiments in which we have measured the DNA-mass ratio, average cell size, and DNA content at different thymine concentrations over the range of conditions where growth is balanced. It is clear that reduction of the amount of thymine causes a progressive decrease in the DNA-mass ratio ($\bar{G}/\bar{M}$) and an increase in average cell size ($\bar{M}$). There appears

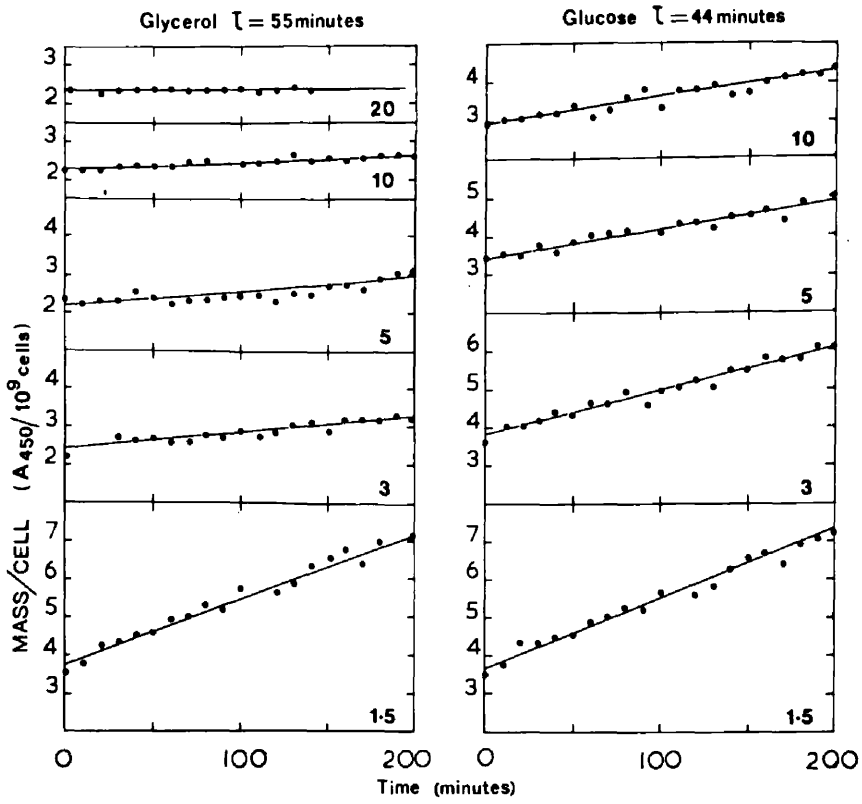

FIG. 5. *Effect of thymine concentration on the rate increase of average cell mass with time of LEB16 grown in media containing glucose and glycerol as carbon source. Thymine concentrations (micrograms per milliliter) are as indicated in the frames.*

TABLE 1. *Average cell size and composition at different thymine concentrations*

Thymine (μg/ml)	DNA/mass (counts per min/$A_{450} \times 10^{-3}$)	C^a (min)	Mass/cell		DNA/cell	
			Predicted[b]	Observed	Predicted[b]	Observed
20	58.64 ± 0.28	41	1.00	1.00	1.00	1.00
5	55.97 ± 0.50	51 ± 3	1.11	1.07 ± 0.01	1.06	1.01 ± 0.01
4	53.47 ± 0.37	60 ± 3	1.22	1.08 ± 0.02	1.12	0.99 ± 0.01
3	52.79 ± 0.34	64 ± 3	1.28	1.15 ± 0.02	1.14	1.03 ± 0.02
2	51.47 ± 0.47	68 ± 3	1.33	1.16 ± 0.02	1.17	1.02 ± 0.01
1.5	49.81 ± 0.59	75 ± 3	1.44	1.24 ± 0.02	1.22	1.04 ± 0.02

[a] Calculated from changes in DNA-mass ratio as in Pritchard and Zaritsky (18).
[b] Calculated by equations (i) and (ii) using C values from column 3 and assuming D = 22 min.

to be very little effect upon the average DNA content per cell ($\overline{G}$). These changes are qualitatively those to be expected if a lowering of the thymine concentration causes an increase in the length of C.

From these data we are able to determine whether there is any effect upon D. Inspection of the equations (i), (ii), and (iii) shows that whereas $\overline{G}$ and $\overline{M}$ are functions of C, D, and τ, $\overline{G}/\overline{M}$ is independent of D. Since the doubling time (τ) is unaffected over this range of thymine concentrations, the $\overline{G}/\overline{M}$ data can be used to quantitate changes in C. The results of these calculations, which are made by a ratio method (18) based upon C of 41 min in 20 μg of thymine per ml (see membrane elution data), are presented in Table 1. We have also calculated predicted values for $\overline{M}$ and $\overline{G}$ for each value of C, assuming that D remains constant at 22 min. Comparison of the predictions and observations indicates that a discrepancy exists between the two sets of data. It could be that our assumption about the constancy of D is incorrect. It is impossible to choose a single value for D such that the predictions and observations about $\overline{M}$ and $\overline{G}$ are equivalent at all thymine concentrations.

These results can be used further to explore the relationship between C and D. We have used the measurements of average cell mass $\overline{M}$ to quantitate changes in $C + D$ arising as a result of the variation in C, and by subtraction

of the estimates of C which we have already calculated (Table 1), it is possible to arrive at an estimate of the size of D in each steady-state condition (Table 2). Calculations of this type, again assuming C and D to be 41 and 22 min, respectively, at the highest thymine concentration, show that as the transit time (C) for a replication fork is extended there is a shortening of the period between termination and division. However, the changes in C and D are not equivalent. The increment in C is larger than the decrease in D. Thus examination of the pattern of DNA synthesis during the division cycle should show changes in the time of occurrence of both initiation and termination. Figure 6 shows a comparison between the observed pattern of DNA synthesis in high (20 μg/ml) and low (2 μg/ml) thymine (see membrane elution data; Fig. 2) and the predicted cell cycle based upon the values of C and D from Table 2. The similarity indicates a correlation between the two sets of data.

Kinetics of transitions between different steady states of growth. The ability to vary the transit time C can be used in another way to investigate whether there is a temporal relationship between the chromosome replication cycle and cell division. For, when a steady-state exponential culture growing in a high thymine concentration is transferred to a lower concentration, there is a transient delay in the rate of arrival of replication forks at the chromosome terminus (18). Hence, if division is timed from an event in the replication cycle other than initiation, it too will be transiently delayed after the "step down." If division is timed from termination, for example, then there should be a delay of D minutes before the alteration in the rate of termination is reflected by a change in the rate of cell division. If division is timed from an earlier event in the replication cycle then the delay will be correspondingly longer.

In Fig. 7 we show the result of such a step-down experiment performed with LEB16. Notice that division does continue at the pre-step rate for approximately 25 min and then decreases, which results in an increase in cell size since the rate of mass synthesis (A_{450}) is unchanged. These kinetics are precisely those predicted if a terminal event of the replication cycle times cell division.

In the complementary step-up experiment (Fig. 8) an acceleration in the rate of cell division is observed. Notice, however, that the

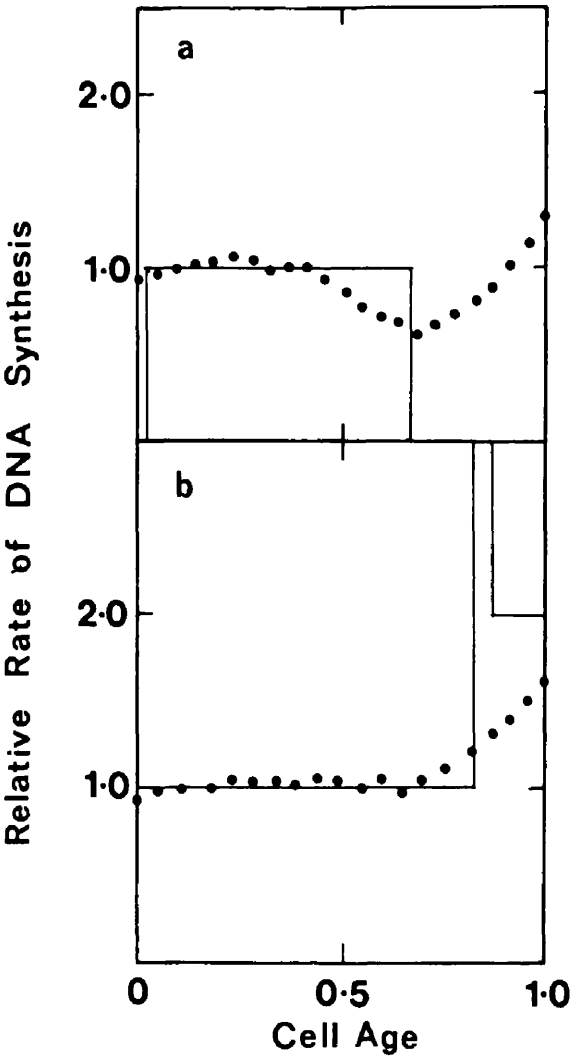

FIG. 6. *Comparison between the observed and predicted patterns of DNA synthesis during growth on high and low thymine. Observed rate of DNA synthesis* (●) *during the cell cycle for cultures in (a) 20 μg of thymine per ml and (b) 2 μg of thymine per ml (data from Fig. 2). Theoretical rate of DNA synthesis* (——) *calculated from values of C and D in Table 2.*

TABLE 2. *Effect of thymine concentration on the length of D*

Thymine (μg/ml)	C^a (min)	Mass/cell (A_{450}/10^9 cells)	$C + D^b$ (min)	D^c (min)
20	41	1.23 ± 0.01	63	22
5	51 ± 3	1.31 ± 0.01	68 ± 2	17 ± 5
4	60 ± 3	1.33 ± 0.01	70 ± 2	10 ± 5
3	64 ± 3	1.41 ± 0.01	75 ± 2	11 ± 5
2	68 ± 3	1.43 ± 0.01	77 ± 2	9 ± 5
1.5	75 ± 3	1.52 ± 0.01	82 ± 2	7 ± 5

[a] See footnote *a*, Table 1.

[b] Calculated from changes in average mass per cell using the relationship:

$$(C + D)_2 = \left[\frac{\tau \cdot \log\left(\frac{\bar{M}_2}{\bar{M}_1}\right)}{\log 2}\right] + (C + D)_1$$

derived from equation (ii).

[c] Calculated by subtracting C from $C + D$.

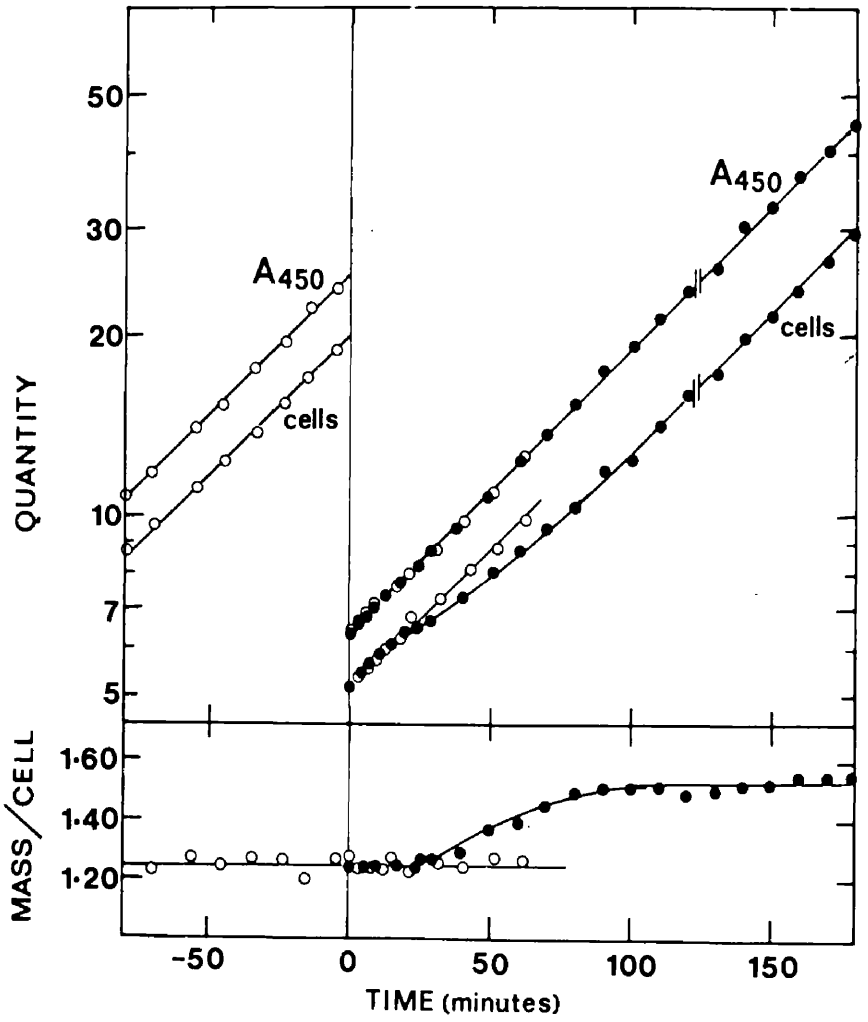

FIG. 7. *The kinetics of increase of absorbance and cell number following a reduction in thymine concentrations. Absorbance A_{450} (10^{-2}) and cell number (10^7/ml) in a culture of LEB16 growing in 6 μg of thymine per ml* (O). *At 0 min the culture was diluted fourfold into fresh prewarmed medium containing the same concentration of thymine* (O) *or no thymine* (●) *to give a final concentration of 1.5 μg/ml. At 125 min the latter culture was diluted twofold into prewarmed medium containing the same concentration of thymine to maintain A_{450} below 0.3. The points after this time are corrected for the dilution. The lower section of the figure shows the same data plotted as average cell mass (A_{450})/10^9 cells.*

period between the times of the transition and the increase in rate is now short (less than 10 min). If the delay period represents the length of *D* in the prestep conditions, these kinetics are consistent with our other findings that a lengthening of *C* is accompanied by a decrease in *D*.

It should perhaps be pointed out that our result from the step-down experiment (Fig. 8) apparently differs from that, previously reported from this laboratory, for a similar experiment performed on *E. coli* 15T⁻ (22). These authors were unable to detect a continuation of the prestep rate of division after transfer to a lower thymine concentration. We have repeated their experiment (Fig. 9) using the same strain, P178, in which we have carefully examined the rate of division immediately after the transition. Our results show that the prestep cell size, and therefore the rate of division, is maintained for a period of about 20 min after the shift. Apart from this difference the shape of the curve is very similar to that obtained by Zaritsky and Pritchard (22). The apparent discrepancy may be due to the fact that previously insufficient samples were assayed immediately after the thymine shift for a firm conclusion to be reached about any possible effect on cell size. We conclude from our data that both *E. coli* B/r T⁻ and *E. coli* 15T⁻ probably behave similarly under these conditions.

These experiments indicate that in steady-state exponential growth conditions the time of cell division is determined by a terminal event of the replication cycle. Thus an apparent paradox exists between these data and those we have already described which support the hypothesis that the time of cell division is triggered earlier in the replication cycle.

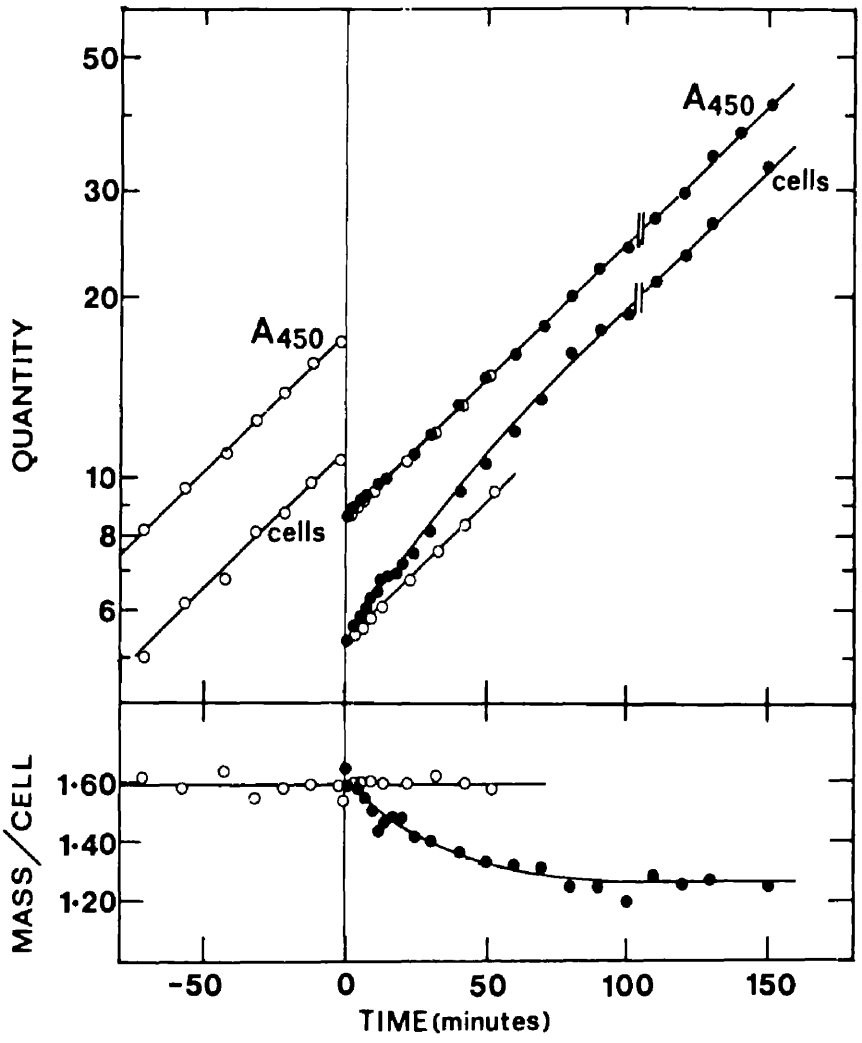

FIG. 8. *The kinetics of increase of absorbance and cell number following an increase in thymine concentration. A_{450} (10^{-2}) and cell number (10^7/ml) in a culture of LEB16 growing in 1.5 μg of thymine per ml* (O). *At 0 min the culture was diluted twofold into fresh prewarmed medium containing the same concentration of thymine* (O) *or 11 μg of thymine per ml* (●) *to give a final concentration of 6.25 μg/ml. At 105 min the latter culture was diluted twofold into prewarmed medium containing the same concentration of thymine to maintain A_{450} below 0.3. The points after this time are corrected for the dilution. The lower section shows the same data plotted as average cell mass (A_{450})/10^9 cells.*

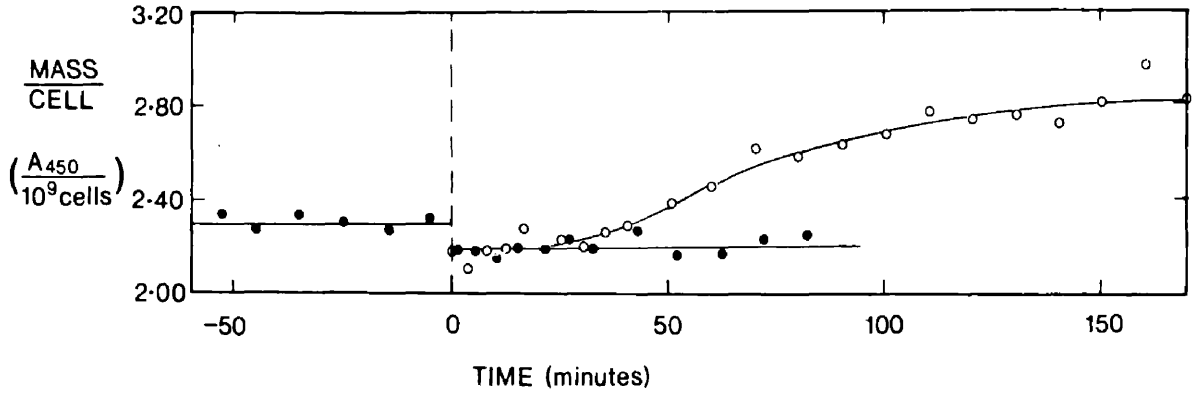

FIG. 9. *Effect of a reduction in thymine concentration on average cell size in E. coli 15T⁻ (555-7) P178. P178 was grown in glycerol medium containing thymine at 2.0 μg/ml* (●) *and supplemented with required amino acids, arginine, methionine, and tryptophan at 50 μg/ml. At 0 min the culture was diluted fivefold with fresh prewarmed medium containing the same concentration of thymine or no thymine* (O) *to give a final concentration of 0.4 μg/ml.*

DISCUSSION

The experiments which we have described were designed to investigate the timing of cell division in relation to the replication cycle. They were not concerned with questions about which events or processes must occur before division is possible. We feel that meaningful questions about temporal relationships are best answered by experiments in which the growth of the cells is disturbed as little as possible.

The elution experiments provide the most direct demonstration that changes in the amount of thymine in the growth medium of thymineless mutants can cause qualitative changes to the length of the replication period *C*. Unfortunately the resolution of this technique is not sufficient for these changes to be quantitated accurately. In addition it is conceivable, in view of recent observations (21), that the incorporation pattern using thymidine as label may be affected by repair synthesis. However, Helmstetter (8) has shown that the pattern of incorporation of thymidine during the division cycle of *E. coli* B/r is essentially the same as the pattern of incorporation of thymine by a thymineless mutant. In addition the DNA-mass ratio measurements, which were made using thymine instead of thymidine, do not experience this source of error and provide independent evidence for a lengthening of the replication period (Table 1). Quantitative estimates of the changes induced in the cell cycle parameters *C* and *D* by thymine limitation can be obtained from measurements of this type on steady-state exponential cultures (Tables 1 and 2) (18, 22). If the assumptions (18, 22) underlying the equations used for these calculations are correct for the conditions under which the measurements are made then the estimates of *C* and *D* that are obtained will not be in error.

The results of the step-up and step-down experiments, in which the culture moves from one steady state of growth to another, lead convincingly to the conclusion that the time of cell division is determined by a late event in the replication cycle. Moreover, the time-determining event occurs at a different cell age in different steady-state conditions. In the step-up transition the period between the timing of division and the division process per se is very short, whereas in the step-down situation the delay is of the order of 25 min. The fact that this difference closely corresponds with estimates of the period between termination and cell division (*D*) in the prestep conditions, as determined from the elution data (Fig. 1 and 2) and steady-state analysis (Table 2), supports the hypothesis that termination provides a signal which determines the time at which the ensuing division will occur. To this extent our data is in good agreement with the original hypothesis (10) and inconsistent with the alternative suggested by others (14, 19).

On the other hand our results also indicate that the length of the period between termination and division is not invariant. It is different in cultures with different transit times, even though they have identical growth rates. Thus the notion of a fixed *D* period (10) is too simple.

To try to understand why the time from termination to division does change in this way it is necessary to ask how changes in *C* influence the cell. We believe that the significant change may be that an increase in *C* causes an increase in cell width, thereby reducing the surface area-volume ratio (22; P. A. Meacock and R. H. Pritchard, unpublished observations). Such an increase in diameter has one obvious consequence; it will increase the amount of wall material needed to construct the septum. This can hardly lead to the difference in *D* that we observe since it is the larger cells that have the shorter *D* (Table 2). A less obvious effect of an increase in cell diameter is that it causes a larger increment in cell volume for any given addition to the cell's length. This may be the significant factor leading to the changes we observe in *D*.

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SUMMARY

The relationship between chromosome replication and cell division has been investigated in a thymineless mutant of E.coli B/r. The investigation is based upon the ability to vary the rate of DNA chain elongation by manipulation of the thymine concentration in the growth medium.

Examination of the changes in average cell mass and DNA content of exponential cultures arising from a reduction in replication velocity suggest that the resulting increase in the replication time C is accompanied by a decrease in the period, D, between termination of the round of replication and the subsequent cell division. Observations on the pattern of DNA synthesis during the division cycle are consistent with this relationship. Nevertheless, the kinetics of transition of exponential cultures moving between steady-states of growth with differing replication velocities provides evidence to support the view that the time of cell division is determined by termination of rounds of replication under steady-state conditions.

Measurements taken from electron micrographs of cells growing with differing replication velocities show that the increase in average cell mass is accommodated by an increase in cell width rather than length. The morphological changes are consistent with the predictions of a model for cell growth whereby the number of sites for

cell elongation, which operate at a constant rate, are equivalent to the number of chromosome termini per cell. Preliminary studies on cell wall murein synthesis indicate an increasing rate with cell age.