STUDIES ON BACTERIAL GROWTH AND REPLICATION

by

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INTRODUCTION

1. I. THE STRUCTURE AND MODE OF REPLICATION OF THE BACTERIAL CHROMOSOME

It was five years after the publication of the work by Watson and Crick (1953) on the double helical structure of DNA that Meselson and Stahl, using a density labeling technique (Meselson and Stahl 1958), established that replication of Escherichia coli DNA is semi-conservative. Subsequently it was shown that the E.coli genome consisted of a single closed circular unit both physically, by autoradiography (Cairns 1963a, b) and genetically (Jacob and Wollman 1961).

The suggestion by Maaløe (1961) that replication of this molecule might be sequential from one or more origins which were fixed from generation to generation had been apparent in the work of Meselson and Stahl (1958). This notion was confirmed by Lark et.al. (1963) by following the kinetics of the conversion of a short radioactively labeled section of the chromosome into hybrid DNA after transfer of the cells into medium containing the density label, bromouracil. The autoradiographs of Cairns (1963) also substantiated this view. However, the interpretation placed on these autoradiographs, that replication proceeded in one direction only, has since been shown to be incorrect (Masters and Broda 1971; Bird et.al. 1972).

The question as to whether replication proceeded from a unique site on the bacterial chromosome (the chromosome origin) was answered in the case of another bacterium, Bacillus subtilis, in a series of experiments by Sueoka and Yoshikawa (Yoshikawa and Sueoka 1963a, b; Sueoka and Yoshikawa 1963, 1965) using both isotopic transfer (to show that replication in this organism was sequential) and marker frequency analysis of exponential and stationary phase cultures. Their analysis depended on the idea that genetic markers situated at the chromosome origin will be present in greater proportion than those at the terminus in exponentially growing cultures if replication is sequential from a fixed origin. Using the Powell age distribution function (Powell 1956), they were able to show how the marker frequency varied with the position of the gene on the chromosome.

In the case of E.coli, there has been some confusion as to the location of the origin and the direction in which replication proceeds. Thus Nagata (1963a, b) has shown that the inducibility of bacteriophage λ doubles at different times in the cell cycle in two lysogenic Hfr strains but shows no such sharp doubling during the cell cycle of a female lysogen. The method used to synchronise the cells has however been critisised (Mitchison 1971) on the grounds that harvesting may cause metabolic disturbances. Several other

reports indicate differences in the pattern of replication in Hfr and F^- strains. Although Berg and Caro (1967) using the bacteriophage P1 to sample the frequency of various genetic markers, found no detectable differences in the gradient of marker frequency in several F^- and Hfr strains, they later concluded (Caro and Berg 1968) using a modification of the P1 transduction assay in which they were able to select for early or late replicating markers, that replication in certain Hfr strains proceeded from a different origin and in a different direction to that in E.coli $15T^-$, B/r and other F^-, F^+ and Hfr strains of E.coli K12. This conclusion was substantiated by Wolf, Newman and Glaser (1968) (see also Wolf, Pato, Ward and Glaser, 1968) using the same technique. Under some conditions and in certain Hfr strains, it was concluded, that replication proceeded from the origin of F replication.

The results of most workers, using marker frequency analysis (Berg and Caro 1967; Caro and Berg 1968, 1969; Wolf, Newman and Glaser, 1968; Wolf, Pato, Ward and Glaser, 1968; Masters, 1970); sequential mutagenesis (Cerda-Olmedo and Hanawalt, 1968; Cerda-Olmedo, Hanawalt and Guerola, 1968) and periodic increases in the potential to produce certain enzymes during the cell cycle (Donachie and Masters, 1966; Helmstetter, 1968; Pato and Glaser, 1968) were all interpreted to show that in a majority of E.coli strains, both

Hfr and F^-, replication proceeded in a clockwise direction from a unique site situated somewhere in the region 50 to 70 minutes on the linkage map (Taylor and Trotter, 1972).

Closer examination of the P1 transduction data, particularly that of Caro and Berg (1968) and Masters (1970) revealed that there was a distinct depression in the marker frequency curve rather than the predicted smooth continuous decrease along the entire length of the chromosome. The possibility that this was due to bidirectional replication of the chromosome from a fixed origin with two replication forks proceeding in opposite directions to a terminus located somewhere in the opposite quadrant was considered by Caro and Berg (1968). Indeed, a more careful analysis of the gradient of marker frequency using several markers and an additional set of E.coli strains in which the lac region was transposed to various positions on the chromosome, revealed a pattern which was entirely consistent with bidirectional replication (Masters and Broda, 1971). This finding was later substantiated by Bird et.al (1972) using a more direct hybridization procedure and more recently by Hohfeld and Vielmetter (1973) using sequential mutagenesis by nitrosoguanidine. Bidirectional replication has since been demonstrated by autoradiography (Prescot and Kuempel, 1972) and biochemically by utilizing the lability of DNA substituted with bromouracil (McKenna and Masters, 1972).

The exact location of the origin is still the subject of controversy. Thus Masters and Broda (1971) suggest that replication is asymmetric with an origin at about 65 minutes, whereas the work of Bird et. al. (1972) suggests that replication is symmetric with an origin situated at 74 minutes. This latter estimate is very similar to that of Hohfeld and Vielmetter (1973) who place the origin at about 75 minutes. The accuracy of these estimates will of course depend critically on the exact location of the genetic markers used, both physically, in terms of their map location and temporally in terms of their relative position in the replication cycle.

1. II. THE CONTROL OF CHROMOSOMAL DNA REPLICATION AND ITS INTEGRATION INTO THE CELL CYCLE

It was suggested by Maaløe that the overall rate of DNA synthesis in E.coli is determined by the frequency of initiation of new rounds of replication and the rate at which each replication fork moves along the DNA duplex (Maaløe 1961). The distinction between initiation and the elongation process became evident from the work of Maaløe and Hanawalt (1961) in which they showed that the increment in DNA observed in E.coli TAU-bar when protein or RNA synthesis was prevented was close to the theoretical value derived on the assumption that rounds of replication which were initiated before the treatment

run to completion but that no new rounds are initiated. This however does not always seem to be the case, since it has been shown by Doudney (1966) that in certain strains, the capacity for DNA synthesis following starvation declines with increasing starvation periods. The experiments of Maaløe and Hanawalt also demonstrated that protein and/or RNA synthesis is required for initiation of chromosome replication and this view was later confirmed by Lark et.al. (1963).

In rapidly growing B.subtilis, the ratio of early to late replicating markers was found to be greater than expected on the assumption that there was one replication fork per chromosome (Yoshikawa et.al. 1964). The value of four which was obtained indicated that there were three growing points per chromosome and demonstrated that new rounds of replication can be initiated before the completion of previous rounds. So-called "dichotomous replication" at fast growth rates has the obvious advantage of reducing the time required for chromosome replication under conditions in which the transit time of a replication fork is fixed (Oishi et.al. 1964).

Pritchard and Lark (1964) showed that "premature" initiation can occur in E.coli after release of the cells from a period of thymine starvation. However, their conclusion that reinitiation occurs at only one of the two chromosome origins has been criticised on the grounds that the deviation of the results from those expected if

initiation were to occur at both origins could be explained if the replication velocity in *thy*$^-$ strains was lower than in *thy*$^+$ strains (Pritchard *et.al.* 1969; Zaritsky and Pritchard 1971).

In fast growing cells, DNA synthesis is continuous. As the growth rate is reduced however, the fraction of cells in the population which are not making DNA at any one time increases (Maaløe 1961; Schaechter, Bentzon and Maaløe 1959). This changing pattern of DNA synthesis suggested that the duration of the replication cycle was similar in cells growing over a range of rates (Maaløe 1961; Pritchard 1966). Maaløe and Kjeldgaard, (1966) suggested that, at a given temperature, the duration of a round of replication was indeed constant. The development of a technique for obtaining synchronous cultures of *E.coli* B/r by selection rather than by induction (Helmstetter and Cummings 1963, 1964) enabled Clark and Maaløe (1967) to test this notion directly. They concluded that the observed doubling in the rate of DNA synthesis some 20 to 25 minutes before division in glucose grown cells corresponded to the initiation of a new round of replication and thus the rate of replication per replicating fork was constant. A more detailed study using a similar technique (Helmstetter and Cooper, 1968; Helmstetter *et.al.* 1968) revealed that the time taken for one round of replication (C) was a constant of about 40 to 45 minutes for cells growing at rates between one and three doublings per hour although limitations in

the technique have prevented an accurate assessment of this time at lower growth rates. This finding does not necessarily mean that the replication velocity of a single replication complex is constant throughout the replication cycle.

The implication, then, is that the overall rate of DNA synthesis is determined, under these conditions, solely by the frequency of initiation of new rounds of replication.

It had been suggested (Pritchard 1965, 1966) on the basis of the observations of Pritchard and Lark (1964) and Oishi et.al. (1964) that initiation of replication may require the attainment of a critical cell mass. The results of Helmstetter and Cooper (1968) indicated that the cell age at which initiation occurs changes progressively with increasing growth rate. It had been known for some time that the average cell size of both E.coli and a closely related bacterium, Salmonella typhimurium, increases exponentially with the growth rate of the culture at a constant temperature (Schaechter et.al. 1958). On the assumption that the size of E.coli changes in the same manner as that of S.typhimurium, Donachie (1968) has shown by combination of these two sets of observations, that per origin cell mass at initiation is "remarkably constant". This would ensure that the frequency with which the culture mass doubles and the frequency of initiation of new rounds of replication are identical. In a formal sense, it constitutes a mechanism for the control of DNA synthesis.

Pritchard et.al. (1969) have proposed a model which would account for a periodic initiation at a constant mass or volume. They argue that initiation must involve a size measuring device. This, they suggest, could be attained by the dilution of a fixed pulse of an inhibitor during growth. When the concentration of this inhibitor reaches a lower threshold, initiation will occur and subsequently another pulse will be produced thus preventing reinitiation until the cell mass has doubled. This formally constitutes a negative control mechanism. Based on the results of Helmstetter and Cooper (1968) and Cooper and Helmstetter (1968), Helmstetter et.al. (1968) have proposed a model which involves the positive control of initiation and is basically a modification of the replicon hypothesis (Jacob et.al. 1963). In this model, it is argued that a critical amount of an initiator substance per chromosome origin must be made during growth before initiation takes place. As yet no critical test has been devised to distinguish between negative and positive control of initiation. These types of model are discussed in more detail later. It should be noted here that the molecular nature of the initiation process is still poorly understood.

The results of Helmstetter and Cooper (1968), in addition to demonstrating that the time taken for one round of replication (C) is constant, indicated that there is a period between the termination of a round of replication and subsequent cell division (D) which is also

a constant and independent of the growth rate of the cells. This period was shown to be approximately 22 minutes. The model which they proposed for the bacterial cell cycle, together with the idea that initiation occurs at a constant cell mass, accounted for the observation that mean cell size was a continuous exponential function of the growth rate (Schaechter et.al. 1958), and the behaviour of cell number, DNA and mass increase when a culture was shifted from a medium which supported a low growth rate to one which supported a higher rate of growth (a shift-up) (Kjeldgaard et.al. 1958). However, it has recently been shown that D can vary under certain circumstances (Zaritsky and Pritchard 1973; Meacock and Pritchard manuscript in preparation) and thus the E.coli cell cycle is certainly more complicated than indicated by the Cooper and Helmstetter model.

III. VARIATION IN THE CHROMOSOME REPLICATION TIME

Although from the work of Helmstetter and Cooper and others, it was evident that the replication velocity (1/C) was constant and independent of growth rate, an earlier observation by Friesen and Maaløe (1965) seemed to contradict this conclusion. When several thymine requiring strains of E.coli were pulsed with various concentrations of ^{14}C-thymine, it was discovered that the rate of incorporation increased with increasing thymine concentration under conditions in which the growth rate was presumably not affected. This

effect the authors stated, could not be attributed to leakiness of the thy⁻ mutation. In addition it was discovered that the internal dTTP levels were higher in certain thy⁺ strains than in their isogenic thy⁻ derivatives (Beacham et.al. 1968a, b). These observations led Pritchard and Zaritsky (1970) (see also Zaritsky and Pritchard 1971) to the conclusion that the replication velocity in thy⁻ strains might be limited by the concentration of thymine in the growth medium. This they demonstrated using both E.coli K12(CR34) and 15T⁻,555-7.Two main methods were used. The first involved measurement of the DNA: mass ratio ($\bar{G}/\bar{M}$) of cultures growing at the same rate but in the presence of different concentrations of thymine.$\bar{G}/\bar{M}$ can be expressed in terms of C (the time taken for one round of replication) and T (the mass doubling time of the culture) and is independent of the period D (Pritchard and Zaritsky 1970):-

$$\bar{G}/\bar{M} = \frac{\tau}{kC\ln 2}(1 - 2^{-C/\tau}) \qquad \text{----------(1)}$$

It does depend however on the cell mass at initiation (the initiation mass), k,which is not accurately known. Changes in $\bar{G}/\bar{M}$ in cells growing at a fixed rate will therefore only yield relative values since one value of C must be assumed. The actual assumed value of C does not significantly affect the calculated change in C (ΔC). The value of k was assumed to be constant and independent of the external thymine concentration. The results presented in the first part of this thesis

lend support to this assumption. Further support comes from the observation that ΔC calculated by this means and that calculated using an entirely different method which is independent of k are in agreement.

The second method depends on the observation that removal of a required amino acid from the growth medium prevents further initiation of rounds of replication (Maaløe and Hanawalt 1961). Assuming that all rounds of replication which are in progress at that time run to completion, the increment in DNA as a percentage of the DNA present before removal of the amino acid, (ΔG), is given by (Sueoka and Yoshikawa 1965):-

$$\Delta G = \left\{ \frac{2^n . n . \ln 2}{2^n - 1} - 1 \right\} 100 \qquad \text{---------- (2)}$$

where $n = C/\tau$. Therefore, at a given growth rate, ΔG will be uniquely defined by C.

Both these methods rely on the validity of the Powell age distribution function for an exponentially growing steady state batch culture (Powell 1956):-

$$f(x) = (\ln 2) 2^{1-x} \qquad \text{---------- (3)}$$

where $0 \leqslant x \leqslant 1$ represents the cell age as a fraction of a generation.

Variation in both $\bar{G}/\bar{M}$ and ΔG with thymine concentration indicated that the replication velocity was indeed dependent on the external thymine concentration. Moreover, as has been stated previously, for a given change in thymine concentration, the values of

ΔC obtained by both methods were in agreement. It was noted however that the relationship between C and the external thymine concentration varied in different strains.

The ability to change the replication velocity by varying the concentration of thymine in the growth medium without a measurable change in growth rate of the cells is utilised in the work presented in this thesis.

1. IV. GENE DOSAGE AND ENZYME OUTPUT

Measurement of changes in the gross macromolecular composition of the bacterial cell at different growth rates has contributed significantly to the present understanding of the control of bacterial cell growth. The relationship between the rates of DNA, RNA and protein synthesis in E.coli and S.typhimurium growing in steady state batch culture at different rates has been well characterised (see Maaløe and Kjeldgaard 1966). In the case of DNA synthesis, current evidence is consistent with the following (at least for cells growing with generation times of between 22 and 65 minutes).

a) the time taken for one round of replication in both E.coli and S.typhimurium (Helmstetter and Cooper 1968; Spratt and Rowbury 1971) is a constant of about 41 - 45 minutes. This is independent of the growth rate of the cells.

b) initiation of replication under normal conditions occurs at a fixed cell mass (Donachie 1968; Pritchard et.al. 1969), although under

certain conditions this is not the case (e.g. Worcel 1970)

and c) replication of the chromosome is sequential from a fixed origin (see section I of this chapter).

It follows directly from the above that in a steady state exponentially growing batch culture, the proportion of the various genes relative to each other and to the cell mass will change with the growth rate of the cells. Thus in both B.subtilis and E.coli (Yoshikawa et.al. 1964; Helmstetter and Cooper 1968) multifork or dichotomous replication has been observed in fast growing cells whereas in slow growing cells, there is a period in the cell cycle during which no DNA is synthesised (Maaløe 1961). At the level of the single cell, changes of this nature must occur continuously.

That changes in gene dosage are important in determining the rate of synthesis of certain enzymes during the cell cycle has been known for some time. It has been shown for instance that the potential to synthesise certain enzymes doubles at a characteristic time in the cell cycle in both B.subtilis and E.coli (Masters and Pardee 1965; Kuempel et.al. 1965; Donachie and Masters 1966). It is generally believed that this reflects the time in the cell cycle at which the respective structural gene is replicated. Thus a doubling in sucrase potential in B.subtilis occurs at the same time as the doubling in sucrase transforming capacity of the DNA (Masters and Pardee 1965), and in E.coli, the doubling in potential to synthesise β-galactosidase can be prevented by inhibiting replication

(Donachie and Masters 1966; Pato and Glaser 1968). Measurement of the basal level of synthesis of alkaline phosphatase in both E.coli and B.subtilis (Donachie 1965; Kuempel et.al. 1965), β-galactosidase in E.coli (Kuempel et.al. 1965) and sucrase potential in B.subtilis (Masters and Donachie 1966) indicates a periodic doubling in their rates of synthesis during the cell cycle. This also seems to be true for fully induced and constitutive β-galactosidase synthesis in E.coli (see Donachie and Masters 1969). As far as can be shown, the time in the cell cycle at which these doublings in rate occur correspond to the times at which the potential to produce the enzyme doubles. It must be noted here however, that since the enzymes are synthesised continuously, the maximum difference in enzyme levels between a system in which the enzyme is synthesised at a continuously increasing rate and one in which it is synthesised at a constant rate which doubles periodically is 6%. In many instances the degree of accuracy of the enzyme assays has prevented this distinction from being made (e.g. Cummings 1965). Data such as this support the view that under conditions in which the degree of repression or derepression per gene remains constant, the rate of enzyme synthesis is proportional to gene dosage (Donachie and Masters 1969). The term gene dosage is however ambiguous. It is used in the literature for at least two different parameters (Maaløe 1969; Donachie and Masters 1969; Stetson and Somerville 1971; Revel 1965) and also encompasses a third:-

a) the first may be called gene dose and can be defined as the number of copies of a specific gene per cell ($\bar{F}$).

b) the second is relative gene dosage and may be defined as the number of copies of a specific gene relative to all genes ($\bar{F}/\bar{G}$).

c) the third I have called gene concentration. This I have defined as the number of copies of a specific gene in unit mass ($\bar{F}/\bar{M}$).

These three parameters vary in different ways during the cell cycle. They also vary in different ways when considered as population averages in steady state batch cultures growing at different rates (see 1. section VIII). The magnitude of these variations in batch cultures with growth rate may be quite considerable. Thus in E.coli with a replication time of 45 minutes, the relative dosage of a gene situated at the origin will increase by 48% as the growth rate is increased from 0.9 to 2.7 generations per hour, whereas the relative dosage of a terminal gene will decrease by 34% as the growth rate is increased over this range. The gene dose, on the other hand, will increase 3.8-fold for a gene situated at the origin and for a terminal gene there will be an increase of 1.6-fold. The corresponding changes in gene concentration for a gene situated at the origin and a terminal gene will be zero and a decrease of 60% respectively. These calculations are based on equations (5), (7), and (11).

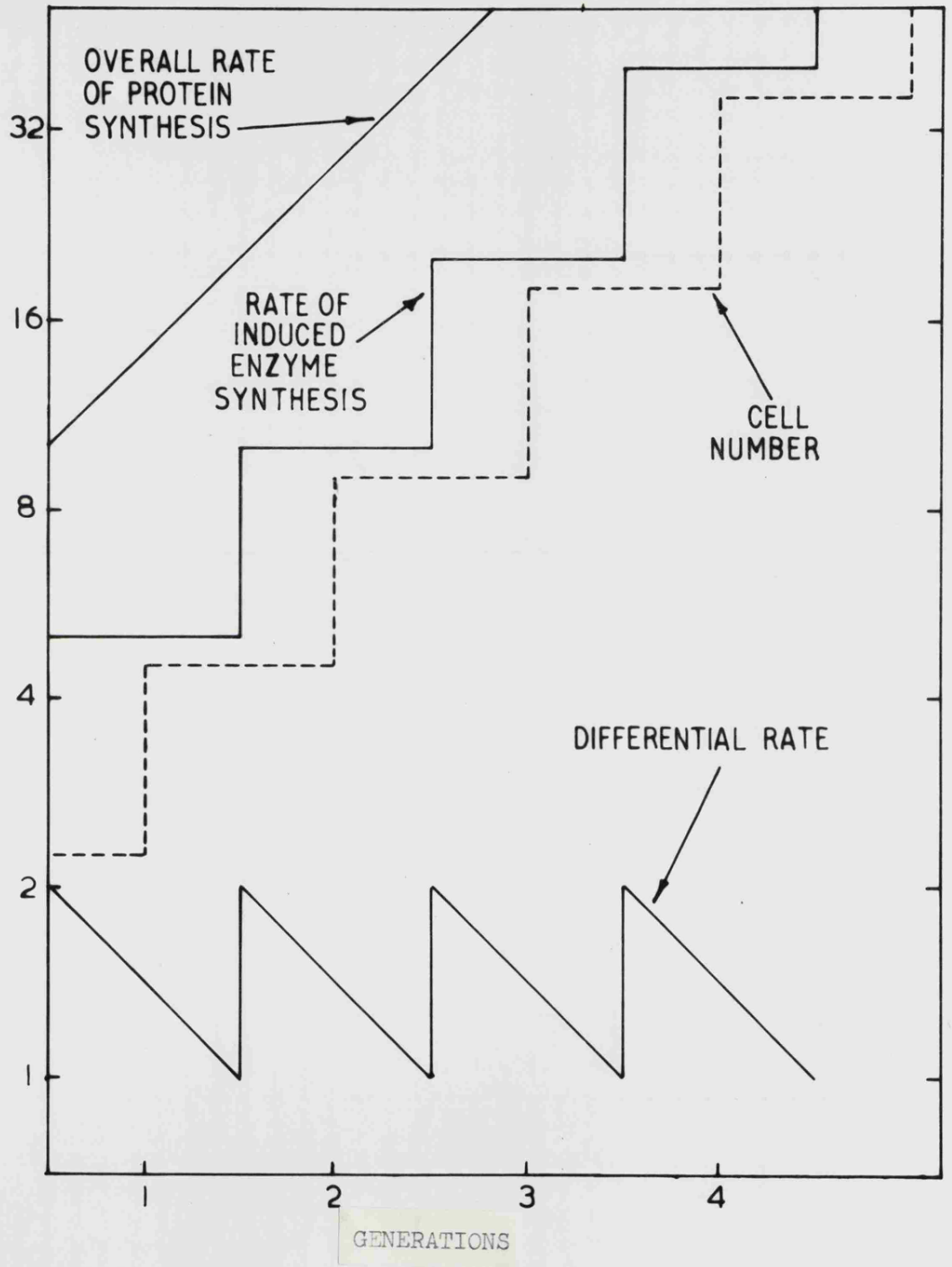
The relative importance of these parameters in specifying the rate of enzyme synthesis under conditions of constant repression or derepression has not been determined. The assessment of enzyme output data in synchronous cultures in this regard is difficult, although it is of interest to note that if, as is generally believed, the overall rate of protein synthesis increases during the cell cycle (Manor and Haselkorn 1967; Ecker and Kokaisl 1969; Kubitschek 1970 and P. Meacock pers. comm.) and the rate of enzyme synthesis per gene is constant, the differential (or relative) rate of enzyme synthesis must decrease continuously until such time as its structural gene is replicated (figure 1). This would suggest that the rate of enzyme synthesis in this case is limited by the number of copies of its structural gene but does not distinguish between the effects of a), b), and c) above.

The activity of basal or constitutively synthesised enzymes in steady state batch cultures growing at different rates is also difficult to assess since it is not possible to rule out variations due to general control mechanisms operating within the cell. It is known for example that several inducible enzyme systems are subject to catabolite repression (de Crombrugghe et.al. 1969) and the degree of this repression is known to be influenced by the composition of the medium in which the cells are grown.

The observation that the activity of certain gene products is

FIGURE 1.

Diagrammatic representation of the theoretical change in the differential rate of synthesis (dE/dP) (Monod et.al. 1952) of an enzyme such as β-galactosidase (bottom curve) whose rate of synthesis increases in a stepwise fashion (centre continuous curve). It is assumed that the overall rate of protein synthesis increases in an exponential fashion (top curve).


OVERALL RATE
OF PROTEIN
SYNTHESIS
RATE OF
INDUCED
ENZYME
SYNTHESIS
CELL
NUMBER
DIFFERENTIAL RATE
32
16
8
4
2
1
1
2
3
4
GENERATIONS

increased in merodiploid strains (Jacob and Monod 1961; Jacob et.al. 1963; Garen and Garen 1963; Pittard and Ramakrishnan 1964; Sparling et.al. 1968; Cassio et.al. 1970; and Stetson and Somervile 1971) also demonstrates the effects of an increase in gene number. Precise quantitation of these data however is difficult for several reasons. In the first place it has not been demonstrated that the output of a chromosomal gene is equivalent to the output of the same gene located on an episome, and secondly, the relative number of copies of the episomal and chromosomal gene is in most cases unknown. A fuller discussion of this data will be found in section 4. VII.

1. V. THE SEX FACTOR F: ITS STRUCTURE AND PROPERTIES

Many species of bacteria, in addition to their chromosome, carry small extrachromosomal genetic elements which are inessential for the viability of the cell. These genetic units are called plasmids. In certain cases they have the ability to integrate into the bacterial chromosome. Elements which are able to undergo integration are known as episomes (Jacob and Wollman 1958). Plasmids may be classified into two major groups according to their ability to promote their own transfer from one cell to another. Those which are able to do this are called infectious, those which are not, are said to be non-infectious plasmids. Infectious plasmids which are able to promote transfer of

genetic material other than their own are known as sex factors. The F particle of E.coli is such a sex factor (Jacob and Wollman 1958). It can exist within a cell as an autonomous unit or it can integrate at a number of loci on the bacterial chromosome. In the former state, the cell is designated F^+ and in this configuration, the F particle is able to transfer chromosomal material with low efficiency into a recipient F^- cell. In the integrated state, transfer of chromosomal material occurs with high efficiency in a polarised manner and cells having an F particle in this configuration are called Hfr.

Rare events can occur in which an F particle acquires certain chromosomal genes. A particle of this type which carried the genes specifying β-galactosidase was first isolated and characterised by Adelberg and Burns (1959) and was termed "F-prime" (in this case, F'lac). It has since been shown that many different chromosomal genes can be carried by F particles (see Low 1972 for a recent review). The model proposed by Campbell for the lysogenization of the bacterial chromosome by bacteriophages such as λ and the generation of transducing phage particles upon induction (Campbell 1962) was used to explain the formation of F-primes. It was able to account for the observation that F-primes generally carry genes with which they were associated in the Hfr state (Scaife 1966) and the generation of an F-prime, F13, which was shown to carry both proximal and distal genes (Broda et.al. 1964).

Two predictions of this model are that, like the bacterial chromosome (Jacob and Wollman 1961; Cairns 1963a, b), the episome should be circular, and that the generation of F-primes must yield a chromosome with a deletion corresponding to the fragment incorporated into the F-prime. Both predictions have been verified. Thus in the strains from which the F-prime factors F13 (Scaife 1966) and F14 (Pittard and Ramakrishnan 1964) were isolated, a chromosomal deletion was demonstrated which corresponded to the fragment incorporated by these F-primes. The circular nature of F-prime particles has been demonstrated both by physico-chemical studies (Freifelder 1968a, b; Freifelder et.al.1971) and by electron microscopy (Martuscelli et.al. 1971). Indeed, the observation that other plasmids such as FcolVcolB-trp-his (Hickson et. al. 1967), several R factors (Cohen and Miller 1969; Silver and Falkow 1970) several col factors (Roth and Helinsky 1967; Bazaral and Helinsky 1968) and many bacteriophages (see Helinsky and Clewell 1971 for a review) also appear to exist as circular structures, suggests that circularity is a requirement for sustained replication of genetic elements within a bacterial cell.

Estimates of the size of F^+ particles by electron microscopy suggest that it is about 2-3% the size of the bacterial chromosome with a molecular weight of between 6.4 and 6.6 x 10^7 daltons (Clowes 1972).

1. VI. THE SEX FACTOR F : ITS REPLICATION

For many types of plasmid, the number of copies per bacterial genome equivalent is small (of the order of 1 to 2). In several other cases including colE1, this number is of the order 10 to 40 (see Clowes 1972). In the former case, plasmid replication is said to be stringent, and in the latter, relaxed. The sex factor F is a member of the former group.

Since the number of F particles per cell is quite small and their inheritance is relatively stable, a mechanism must exist which couples the frequency of F replication to the growth rate of the host cell.

Jacob et.al. (1963) proposed that stable, autonomously replicating units (which they called replicons) such as the bacterial chromosome and the F-particle, were attached to a membrane site. The attachment site would then control the initiation signal for both chromosome and F replication and would double once per generation. Stable transmission of both chromosome and episome complements, they supposed, would occur from one generation to the next. Although the model would account for the occurrence of the phenomenon of incompatibility between autonomous plasmids (that is, the inability of two closely related plasmids to reside in a single cell) (DeHaan and Stouthamer 1963) by supposing competition for one attachement site, the observation of incompatibility between two F particles when one is

in an Hfr state and the other a superinfecting autonomous F (Dubnau and Maas 1968) is difficult to explain (Pritchard et.al. 1969). This observation would suggest that in Hfr cells, the F particle is attached to the same membrane site as it is in F^+ or F' cells. F replication in most Hfr strains, however, seems to be under chromosomal control (section 1. I). It would thus be necessary to assume that membrane attachment sites were not the controlling element in replication but that another level of control must operate on the replication process.

Genetic evidence concerning the control of F replication is difficult to assess. Mutants have been isolated in which the ability for either F replication or the initiation of chromosome replication is impaired at high temperature (Jacob et.al. 1963). The mutations affecting episomal replication can be located either on the episome or on the host chromosome. Other mutants have been isolated in which the thermolability of a mutant F particle is suppressed. The locus concerned is located on the host chromosome (Yamagata and Uchida 1972). In addition, it has been observed that a resident F particle can suppress certain so-called chromosomal initiation mutants (Hirota et.al. 1970) by integration into the chromosome. This phenomenon has been called integrative suppression (Nishimura et.al. 1971). The replication of F is therefore not strictly independent of chromosomal control and under

some circumstances chromosome replication seems to be controlled by an integrated episome.

The nature of this interrelationship is at present unclear. It is known however, that unlike the episome col1BM trp,lac. (Zeuthen, Morozow and Pato 1972), F replication occurs at a specific time in the cell cycle (Nishi and Horiuchi 1966; Donachie and Masters 1966) as judged by the observed doubling in the potential to synthesise β-galactosidase specified by an F'lac particle and that this time can be separated from the time in the cell cycle at which the chromosomal lac gene is replicated (Donachie and Masters 1966). The assumption in work of this nature is that the doubling in the lacz gene reflects the time at which the whole Flac factor replicates. If F replication proceeds at the same rate as that of the chromosome, one round of F replication would take from 2 to 3 minutes and the above assumption would be valid.

Measurements of the timing of F replication in cultures of E.coli B/r synchronised by the Helmstetter and Cummings technique (Helmstetter and Cummings 1963, 1964) and growing at different rates have been interpreted in different ways. Zeuthen and Pato (1971) have obtained evidence suggesting that at certain growth rates, initiation of chromosome replication and F replication occur at different times in the cell cycle. This would imply that F replication cannot be directly coupled to initiation of rounds of chromosome replication. They

concluded that the F'lac factor replicates about one half to two thirds of the way through the cell cycle over a wide range of growth rates. On the other hand, both Cooper (1972) and Davis and Helmstetter (1973) have concluded that, in the same way as chromosome initiation, F replication occurs at a fixed cell mass. Using synchronous cultures which were undergoing a shift-up (Kjeldgaard et.al. 1958), Cooper concludes that F replication occurs slightly before chromosome initiation. Davis and Helmstetter on the other hand concluded that F replication occurs at the same time as chromosome initiation under their conditions (for a more complete discussion of these results see section 4. V).

Further experiments by Zeuthen and Pato (1971) using F'lac/Δlac and F^-/lac^+ derivatives of a temperature sensitive initiation type mutant (dnaC) indicated that the cessation of F replication occurs at about the same time as the cessation of chromosomal DNA synthesis although the culture mass continues to increase. This result was taken to indicate that F factor replication might be temporally associated with replication of terminal markers on the chromosome and might suggest that F factor replication is coupled to termination of a round of chromosome replication.

Bazaral and Helinski (1970) have investigated the kinetics of F replication and chromosome replication in E.coli CR34 F^+ during inhibition of protein synthesis by amino acid starvation. Their

results are similar to those of Zeuthen and Pato using the dnaC type mutant, and were taken to indicate that initiation of rounds of replication of F DNA is depressed during the amino acid starvation although not as rapidly or to the same extent as initiation of chromosome replication. These apparent differences in the control of initiation of the two replicons have led to the conclusion that chromosomal DNA synthesis is required for F factor replication. The results are consistent with a requirement for termination of a round of chromosome replication for F DNA synthesis but do not rule out the possibility that F replication occurs coincident with initiation of chromosome replication or at a fixed cell mass.

The proportion of F DNA in total DNA of E.coli C600 was however found to fall with increasing growth rate (Collins and Pritchard 1973). This observation is not consistent with the notion that F replication occurs coincident with initiation of chromosome replication or at a constant cell mass.

1. VII. PRESENT WORK

The purpose of the work presented in this thesis was twofold. In the first place, I have tried to determine what factors are important in determining the maximal rate of gene output (see section 1. IV), and secondly, using the same technique, I have investigated the possibility that F replication is coupled directly to some stage of the

chromosome replication cycle.

a) Gene dosage, relative gene dosage, gene concentration and gene output.

In section 1. IV, I described three parameters involving gene number. These were:- gene dosage, relative gene dosage and gene concentration. I indicated that these three parameters varied in different ways both during the cell cycle and with growth rate in steady state exponentially growing batch cultures. An assessment of the importance of these in determining the fully repressed or derepressed rate of synthesis of gene products is difficult. Unfortunately, the effect of changes in these parameters with growth rate may be modulated by general control mechanisms such as catabolite repression operating within the cell.

The changes in chromosome configuration which occur on increasing the growth rate are mediated by an increased frequency of chromosome initiation in thy^+ cells with a constant replication time, C. If the frequency of initiation is kept constant, the same changes should occur with increasing replication times. Both would be predicted to increase the number of replication forks per chromosome. This is shown diagrammatically in figure 2 a and b. It has been shown, that the replication time in thy^- cells can be varied without measurably affecting the mass doubling time of the culture (Pritchard and Zaritsky 1970; Zaritsky and Pritchard 1971). Under these conditions, the effect of changes in

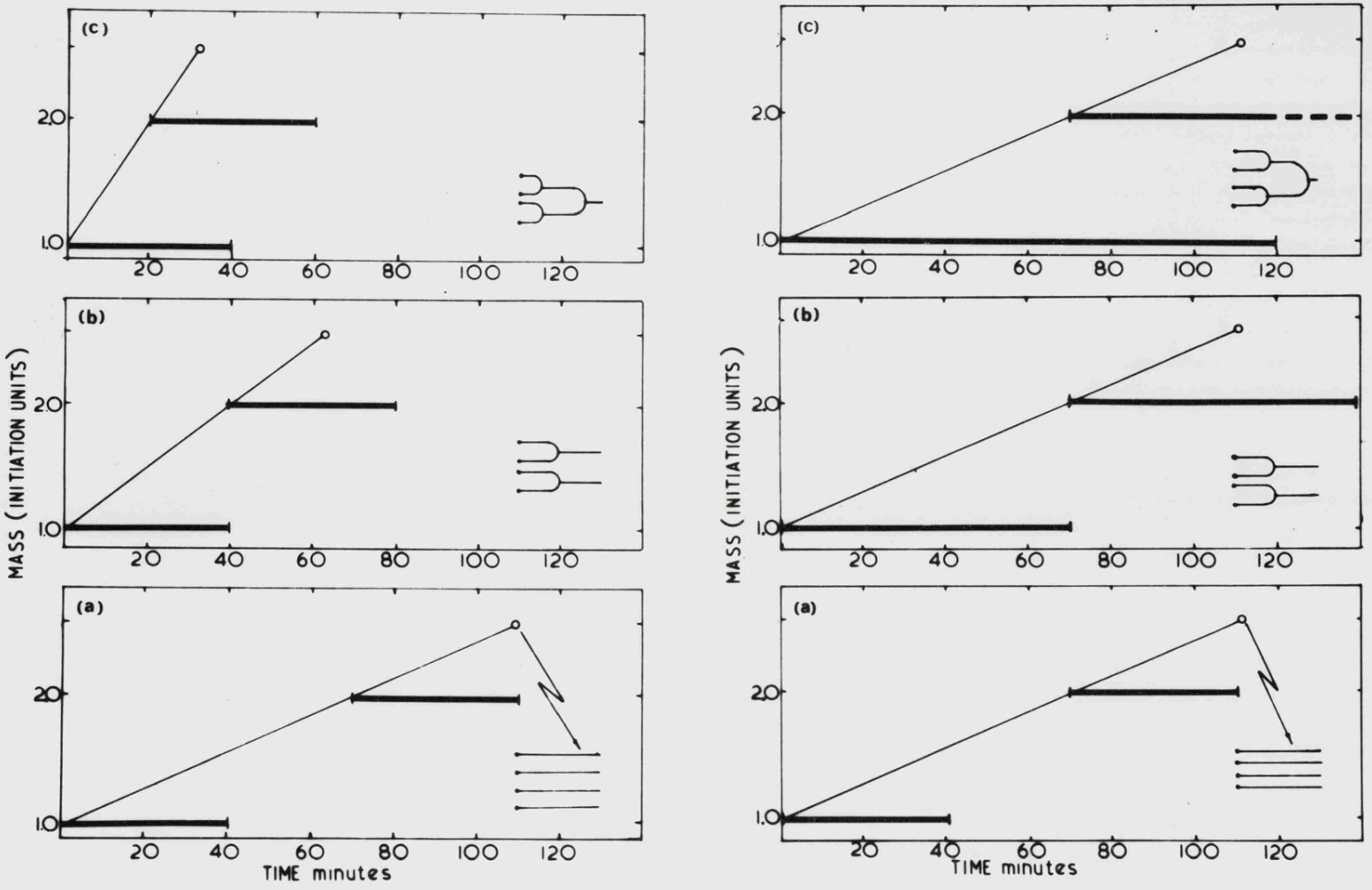
FIGURE 2.

DIAGRAMMATIC REPRESENTATION OF THE RELATIONSHIP BETWEEN MASS DOUBLING TIME, REPLICATION FORK TRANSIT TIME AND CHROMOSOME CONFIGURATION.

In each panel at $t = 0$, there is one initiation mass unit per chromosome origin. Initiation of a new round of replication therefore occurs at this time, and again when the mass reaches two initiation mass units. The thick horizontal lines represent the transit time of a replication fork. In the bottom right hand corner of each panel, I have shown the chromosome configuration which would occupy 3 initiation mass units (since replication is bidirectional, only half of the chromosome is shown). Cell division is ignored.

The three left hand panels demonstrate the effect of decreasing the mass doubling time from 70 minutes (panel a) to 20 minutes (panel c) whilst maintaining the transit time at 40 minutes. The three right hand panels show the effect of increasing the transit time from 40 minutes (panel a) to 120 minutes (panel b) when the mass doubling time is maintained at 70 minutes.

Both operations result in an increase in the ratio of origin to terminal genes. It can be seen that whereas the concentration of genes situated at the origin remains constant, the concentration of terminal genes decreases. Since, under these conditions, the DNA concentration (DNA:Mass ratio) decreases, the relative dosage of genes situated at the origin must increase and that of terminal genes must decrease. This is represented more formally in figures 4 and 5.


(c)
(b)
(a)
MASS (INITIATION UNITS)
TIME minutes
1.0
2.0
20
40
60
80
100
120

gene dosage, relative gene dosage and gene concentration on gene output can be determined without the complicating and indeterminate changes in cell metabolism brought about by changes in growth rate.

b) F replication.

Data presented in the first part of this thesis demonstrates that the output of a gene under conditions in which the degree of repression or derepression remains constant, is proportional to the concentration of that gene. Further, the magnitude of the change in concentration of any gene, for a given change in replication velocity in cells growing at a fixed rate, is a function of the distance of the gene from the origin of replication (see section 1. VIII). Comparison of the magnitude of the change in concentration of a chromosomal lac operon with the change in concentration of the same operon located on an F-prime factor, in cells undergoing a given change in replication velocity and growing at the same rate, should enable an estimation of the temporal position of F replication in the cell cycle, if it is directly coupled to the chromosome replication cycle. The results are discussed in the light of other data concerning the timing of F replication in the cell cycle (Cooper 1972; Davis and Helmstetter 1972; Zeuthen and Pato 1971), the relationship between F DNA and chromosomal DNA in batch cultures growing at different rates (Collins and Pritchard 1973), and the relative contribution to total enzyme from episomally located genes in merodiploid strains

(e.g. Revel 1965).

VIII. THEORETICAL

In section IV, I defined three different parameters which can be used to describe different aspects of gene number. These were:-

gene dose: the number of copies of a specific gene per cell.

gene concentration: the number of specific genes per unit mass.

and relative gene dose: the number of copies of a specific gene relative to all genes.

Each of these parameters refers to the average value in a batch culture which is in a steady state of growth.

The equation relating gene dose ($\bar{F}$) to the mass doubling time of the culture (τ) and the age in the cell cycle at which the specific gene is replicated (a/τ) is given by:- (Collins and Pritchard 1973)

$$\bar{F} = 2^{1-a/\tau} \qquad \text{----------(4)}$$

This is essentially a modified form of the equation derived by Sueoka and Yoshikawa (1965) and relies on the validity of the Powell age distribution function (equation 3). Here a has the limits: $\tau-(C+D) \leqslant a \leqslant \tau - D$, where D is the period between termination of a round of replication and subsequent cell division (Cooper and Helmstetter 1968).

Put in a more general form, equation (4) becomes:-

$$\bar{F} = 2^{(C\,[1-x]\,+D)/\tau} \qquad \text{---------- (5)}$$

where x is the distance of the gene under consideration from the origin of replication as a fraction of the chromosome length (or half the chromosome length if replication is bidirectional (Masters and Broda 1971; Bird et.al. 1972)). Thus for a gene at the origin, x = 0, and for a gene at the terminus, x = 1.

Since it has been shown that the average cell mass ($\bar{M}$) varies with the mass doubling time over the range 20 mins to 70 mins, according to the following equation:- (Pritchard et.al. 1969)

$$\bar{M} = k.2^{(C+D)/\tau} \qquad \text{---------- (6)}$$

where k is a constant (and is a measure of the initiation mass (Donachie 1968)). It follows that the gene concentration ($\bar{F}/\bar{M}$) can be found by dividing (5) by (6). This gives:-

$$\bar{F}/\bar{M} = \frac{2^{-Cx/\tau}}{k} \qquad \text{---------- (7)}$$

The change in gene concentration (Δgc) for a given change in C with τ constant, is then:-

$$\Delta gc = (\bar{F}/\bar{M})_2 / (\bar{F}/\bar{M})_1 = 2^{(C_1-C_2)x/\tau} \qquad \text{---------- (8)}$$

Similarly, the change in gene concentration for a given change in τ (with C constant) is given by:-

$$\Delta gc = 2^{Cx(1/\tau_1 - 1/\tau_2)} \qquad \text{---------- (9)}$$

The predicted increase in gene concentration for an increase in replication velocity (a decrease in C) on the basis of equation (8), plotted against the map position of the gene is shown in figure 3. Note that Δgc increases exponentially as a function of the distance of the gene from the origin. The slope of the curve depends on the difference in replication velocity and not on the absolute values.

To obtain the corresponding expressions for relative gene dosage, it is necessary to know the average amount of DNA per cell in genome equivalents ($\bar{G}$). This relationship has been derived by Cooper and Helmstetter (1968) and is given by:-

$$\bar{G} = \frac{\tau}{C \ln 2} \left(2^{(C+D)/\tau} - 2^{D/\tau}\right) \qquad \text{---------- (10)}$$

Hence the relative gene dosage can be obtained by dividing (5) by (10):-

$$\bar{F}/\bar{G} = \frac{C \ln 2 . 2^{C(1-x)/\tau}}{(2^{C/\tau} - 1)} \qquad \text{---------- (11)}$$

Treating equation (11) in the same way as (7) to obtain an expression for the change in relative gene dosage for a given change in replication velocity, we obtain:-

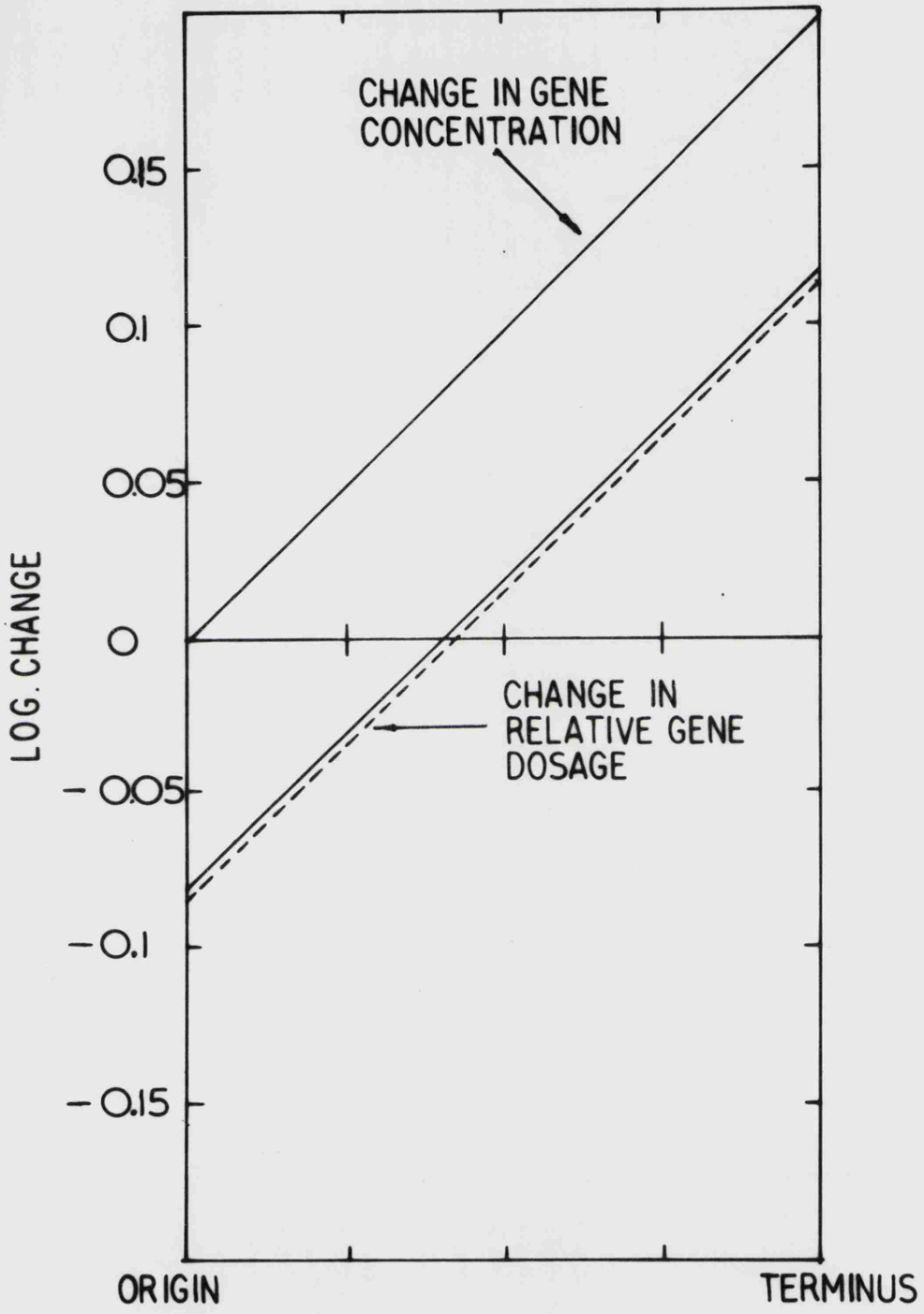
$$\Delta rd = (\bar{F}/\bar{G})_2 / (\bar{F}/\bar{G})_1 = \frac{C_2(2^{C_1/\tau} - 1) . 2^{(C_2 - C_2)x/\tau}}{C_1 (2^{C_2/\tau} - 1)} \text{ ----- (12)}$$

And similarly, for a given change in :-

FIGURE 3.

PREDICTED CHANGE IN GENE CONCENTRATION AND RELATIVE GENE DOSAGE AS A FUNCTION OF GENE LOCATION IN A STEP-UP EXPERIMENT.

The curve for the change in gene concentration has been calculated from equation (8) using the following values: ΔC = 34 minutes; τ = 55 minutes. The curve for the change in relative gene dosage has been calculated from equation (12) (the broken line represents the change in relative gene dosage for a ΔC of 34 minutes from 81 to 45 minutes, and the solid line corresponds to a ΔC of 34 minutes between 101 and 65 minutes).


CHANGE IN GENE CONCENTRATION
CHANGE IN RELATIVE GENE DOSAGE
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
LOG. CHANGE
ORIGIN
TERMINUS

$$\Delta rd = \frac{\tau_1 (2^{C/\tau_1}-1)}{\tau_2 (2^{C/\tau_2}-1)} \cdot 2^{C(1/\tau_1 - 1/\tau_2)} \cdot 2^{Cx(1/\tau_2 - 1/\tau_1)} \quad \text{----- (13)}$$

The logarithmic form of equation (12), plotted in figure 3 (lower curve), has an intercept which is a function of C_1, C_2 and τ. The slope is the same as that for the gene concentration curve (i.e. $(C_1-C_2)\log 2/\tau$). It should be noted however, that there is an important difference. The null point, or point of zero change, will occur for a gene near the middle of the replicon in the case of relative gene dosage, but at the origin for gene concentration.

Figure 2 shows diagramatically, the equivalent effects of increasing the mass doubling time or decreasing the transit time, on the configuration of the chromosome. Both, decrease the ratio of chromosomal origins to termini and, in a sense, increase the degree to which the population average chromosome is replicated. Qualitatively, this has the effect of increasing the concentration and relative dosage of terminal markers and at the same time, decreasing the relative dosage of markers situated at the origin. The concentration of origin markers on the other hand, if initiation occurs at a constant cell mass, will remain constant. Quantitatively, the effects of variation in both C and τ on gene concentration are very similar (figure 4a, b). Their effect on relative gene dosage are also comparable (figure 5a, b). The equivalence of τ and C on gene concentration depends on both the value and the constancy of the initiation mass (k). Relative gene

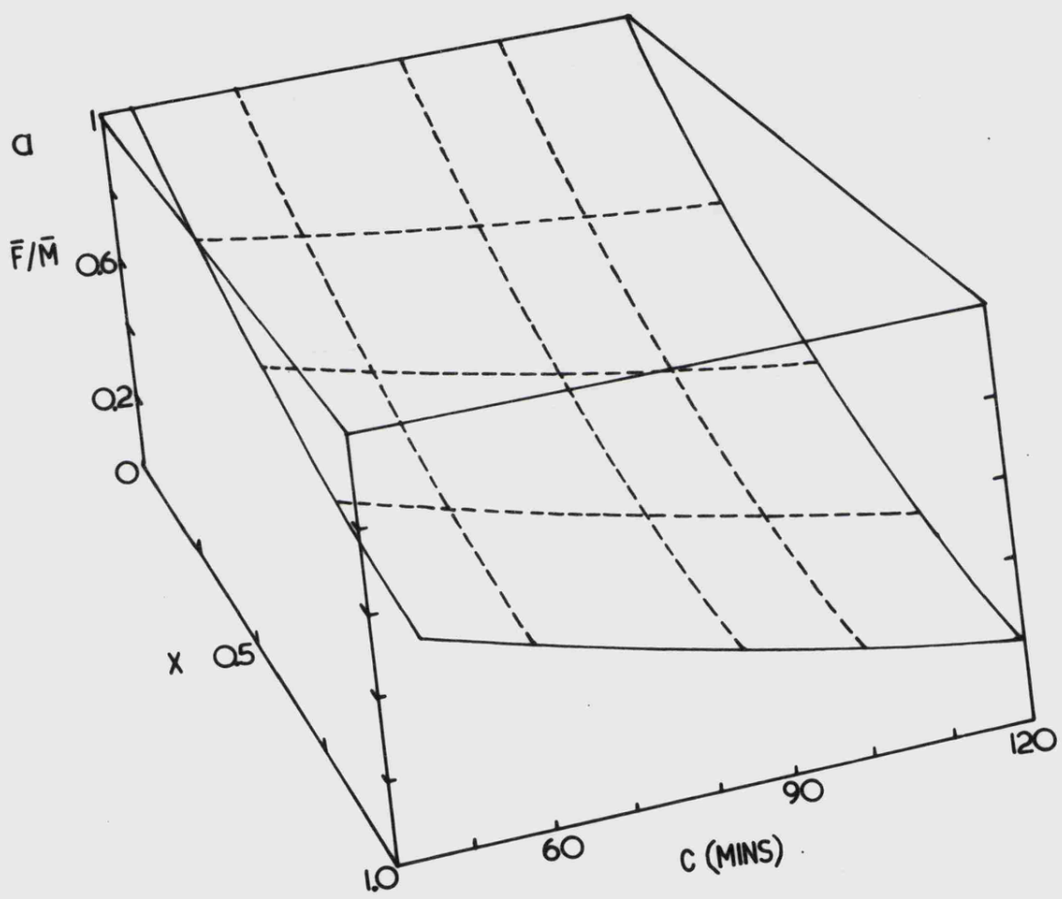
FIGURE 4.

a RELATIONSHIP BETWEEN GENE CONCENTRATION, GENE POSITION AND TRANSIT TIME.

The curve shown was calculated from equation (7) by setting $k = 1$, $\tau = 55$ minutes and varying C. Note that the concentration of genes situated at the chromosome origin ($x = 0$) remains constant and independant of C. Note also that the "sensitivity" of gene concentration to changes in C becomes more pronounced as $x \rightarrow 1$ (i.e. the closer the gene is to the terminus).

b RELATIONSHIP BETWEEN GENE CONCENTRATION, GENE POSITION AND MASS DOUBLING TIME.

The calculations were as in figure 4a with $k = 1$. In this case, C was given a value of 45 minutes, τ varied. It can be seen that the concentration of genes at the origin is also independant of τ, and that the "sensitivity" of gene concentration to changes in τ increases the closer the gene is to the terminus.


a
1
$\bar{F}/\bar{M}$
0.6
0.2
0
X
0.5
1.0
60
90
120
C (MINS)

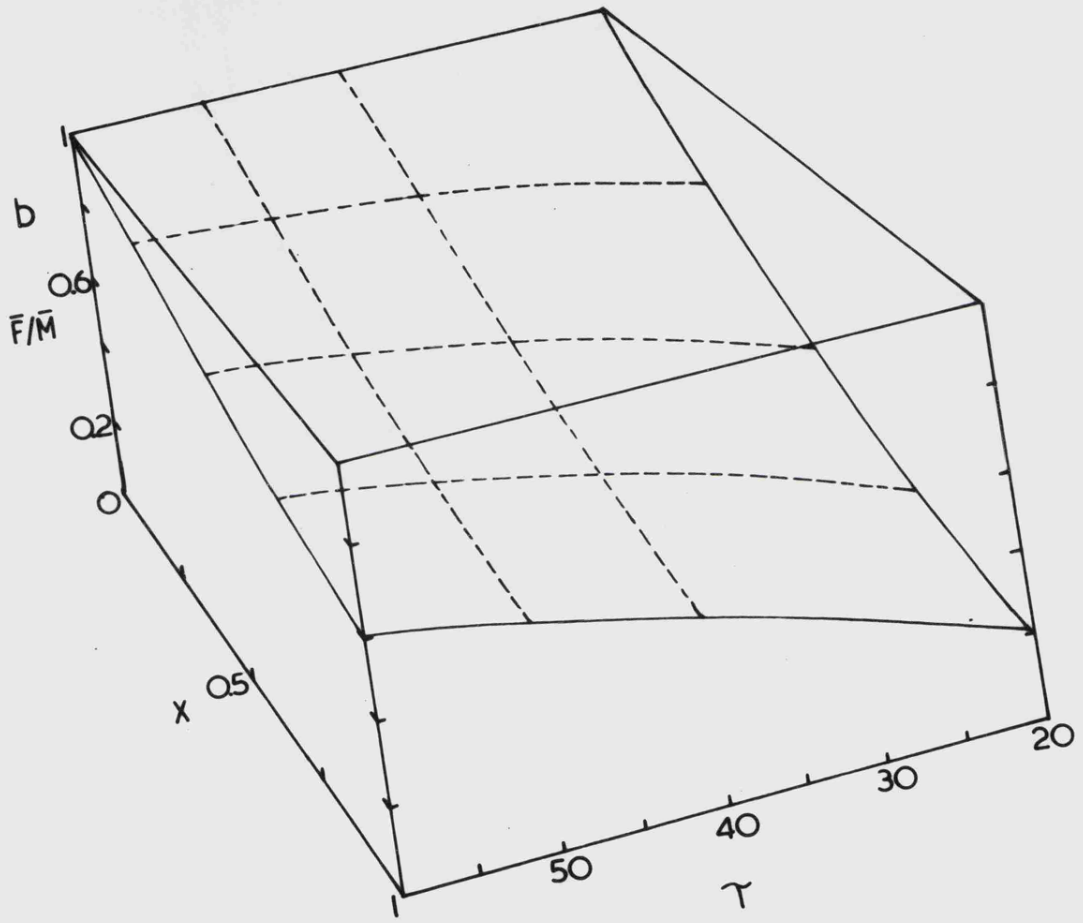

1
b
0.6
F̄/M̄
0.2
0
x
0.5
1
50
40
30
20
τ

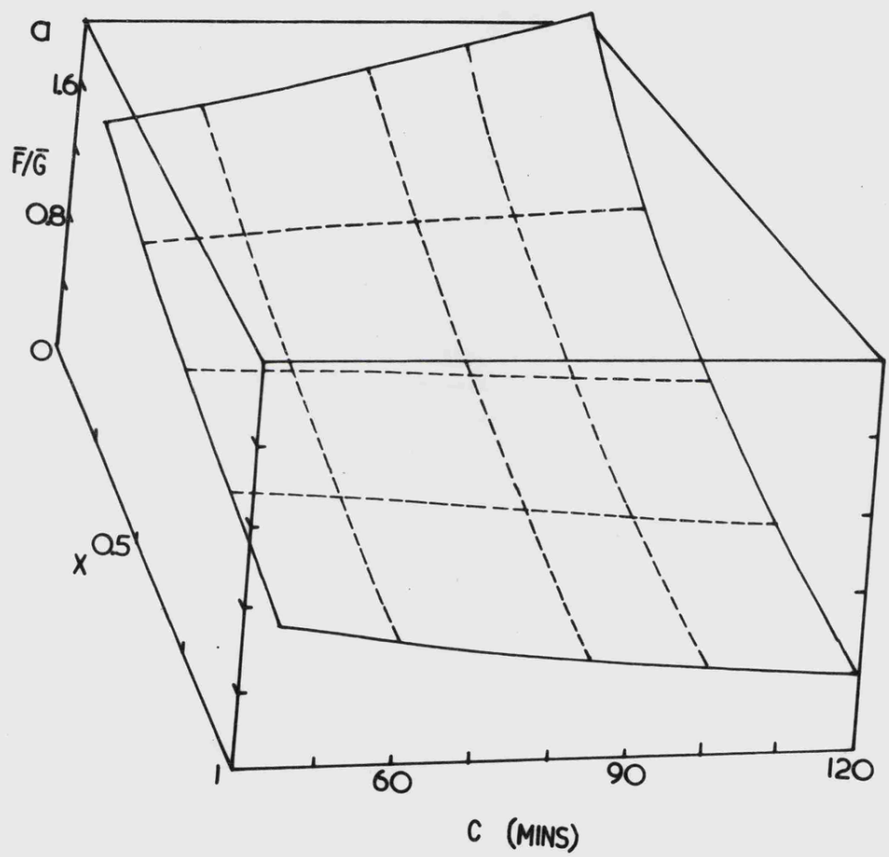
FIGURE 5.

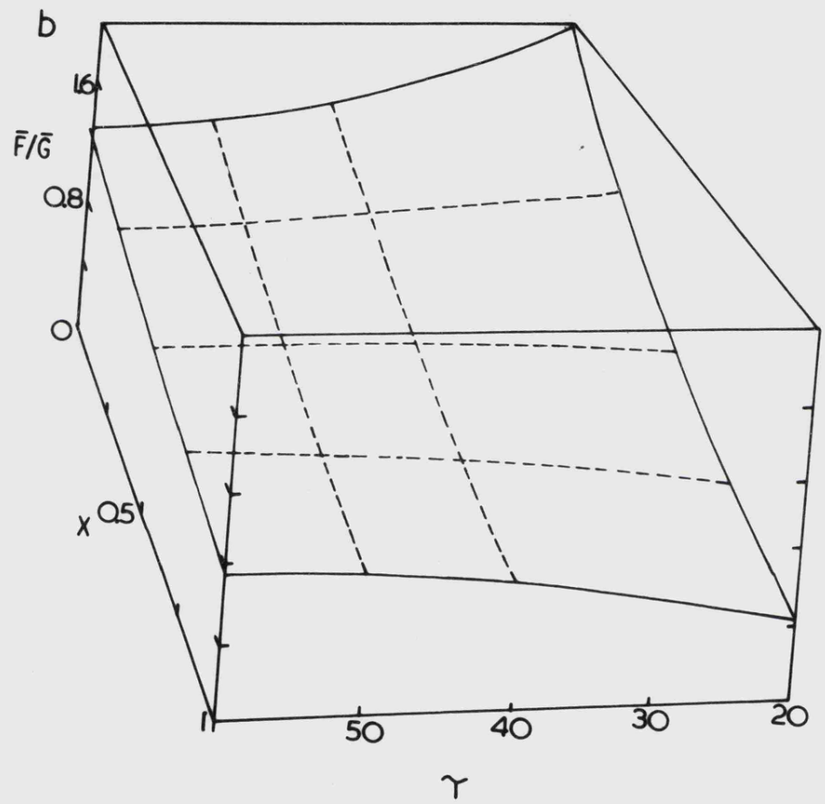
a RELATIONSHIP BETWEEN RELATIVE GENE DOSAGE, GENE POSITION AND TRANSIT TIME.

The curve was calculated using equation (11), setting Υ = 55 minutes. It can be seen that in contrast to gene concentration, the relative dosage of genes situated at the chromosome origin is not independant of C. Moreover, there is a slow but distinct reversal of the effect of changes in C on the relative gene dosage as $x \rightarrow 1$.

b RELATIONSHIP BETWEEN RELATIVE GENE DOSAGE, GENE POSITION AND MASS DOUBLING TIME.

As for figure 5a but giving C a value of 45 minutes and varying Υ. Again, note that the relative dosage of genes situated at the chromosome origin is not independant of Υ, and the reversal of the effect of changes in Υ on the relative gene dosage as $x \rightarrow 1$.


a
1.6
F/G
0.8
0
X 0.5
1
60
90
120
C (MINS)


b
1.6
$\bar{F}/\bar{G}$
0.8
0
x 0.5
1
50
40
30
20
Υ

dosage is however, independent of this.

Gene dosage (equation 5) is more difficult to quantify. It depends on the poorly understood parameter, D. At least in wild type E.coli B/r, this period is believed to remain constant over a range of cell growth rates, and has a value of 20 to 25 minutes at 37°C (Helmstetter and Cooper 1968). The relationship between C and D would also seem to remain constant over a range of temperatures (Pierucci 1971).

Evidence is now accumulating that in some thymine requiring strains, the magnitude of D changes with C (Zaritsky and Pritchard 1973). In B/r for instance, D decreases for increasing values of C (Meacock and Pritchard manuscript in preparation). In view of these complications concerning D, I have not attempted to investigate gene dosage in the same way as relative gene dosage or gene concentration. However, the observation that under certain conditions, the cells used in this study increase in size continuously (see figure 17a, and b) has enabled me to rule out gene dosage as a factor involved in gene output.

2. MATERIALS AND METHODS

2. I. BACTERIAL STRAINS

NUMBER	GENOTYPE	SOURCE
P162-8	*thyA, dra, drm, lacy, thr, leu, thi, str*^{s}	R.H. PRITCHARD
RP282/F^{-}	*lac, thi, str*^{s}	R.H. PRITCHARD
SA85	*lac, ilv, trp, pho, str*^{r}*, tna, bgl*$^{-}$	S. AHMAD
SA85/1	" " " " " " *bgl*$^{+}$	
JC3272	*his, trp, mal, gal, lys, lacX74, str*^{r}, /KLF23 (Ftrp)	P.MEACOCK
RP273	*lacy, pro, arg, thi, recA, str*^{r}, /Flac	R.H. PRITCHARD
AT3095	*trpR65, cysB14, tna4, phoS1, thi, str*^{r}	R. BUXTON

MC5 is a *lacy*$^{+}$ derivative of P162-8 obtained as a spontaneous revertant.

MC6 is a *pro*$^{-}$ mutant of MC5 obtained by 2-amino purine mutagenesis followed by penicillin enrichment.

MC9 contains a deletion of the *lac* operon as judged by its inability to form β-galactosidase and its low reversion frequency (< 1 in 2×10^{9}). It was derived by P1 transduction using RP282 as donor and MC6 as recipient.

MC10 is an Flac derivative of MC9 obtained by infection from RP273.

MC11 was obtained from MC5 by successive reversion at the *thr*, *leu*, and *thi* loci.

MC17 is a tna$^-$ mutant of MC11. It was derived by P1 transduction using SA85/1 as donor. In order to enable a selection to be made, SA85 was first made bgl$^+$ by selection of clones that were able to grow on Aarb.sal.plates. Using this as a donor, bgl$^+$ transductants were selected by their ability to grow on Aarb.sal. plates. These were then screened for co-transduction of the tna locus.

MC32 is a trpR$^-$ mutant of MC17. It was obtained by P1 transduction using AT3095 as a donor.

MC84 is a KLF23 (Ftrp) derivative of MC17. It was obtained by infection from JC3272/KLF23.

MC85 is a KLF23 derivative of MC32, obtained by infection from JC3272/KLF23.

2. II. MEDIA

Minimal(pH=7.0-7.2): contained, Na_2HPO_4(0.7%), KH_2PO_4(0.3%); NaCl(0.5%); NH_4Cl(0.1%); $CaCl_2$(0.002%) and $MgSO_4$(0.02%).

Glycerol minimal medium contained glycerol at 0.4%.

This medium was supplemented with 0.2% acid hydrolysed casein(Difco) (glycerol cas medium) or with the following L-amino acids at 40µgm/ml: alanine, arginine, aspartate, cysteine, glutamate, histidine, isoleucine, lysine, methionine phenylalanine, proline, tyrosine, valine (glycerol amino acid medium). Where necessary, threonine(40µgm/ml), leucine(40µgm/ml) and thiamine(20µgm/ml) were added. Thymine was added to give the concentration

shown in the text.

Nutrient broth: contained 2.5% Oxoid No.2 nutrient broth supplemented with 40μgm/ml thymine.

Tryptone broth:(pH=7.0) contained 1% oxoid tryptone and 0.5% NaCl

Minimal agar: the minimal medium was solidified with 1.5% Davis agar. Carbon sources were added at 0.4%, amino acid supplements at 40μgm/ml and thymine to the required concentration.

Nutrient agar: nutrient broth was solidified with 1.25% Davis agar.

The Z-agar plates used in the transduction procedures contained, in addition, $CaCl_2$(2.5mM) and glucose (0.1%).

Tetrazolium plates contained, in addition to the nutrient agar, tetrazolium chloride(0.005%).

EMBO phage plates were made by the addition of glucose (0.125%) and EMB broth (Difco) (1.25%) to nutrient agar.

Soft agar: nutrient broth was solidified with 0.6% Oxoid No. 1 agar and contained 2.5mM $CaCl_2$.

Aarb.sal. and ABsal.agar: Selection of bgl^+ mutants was performed on Aarb.sal plates. These were minimal agar containing arbutin and salicin, both at 0.25%.

The ABsal plates used for screening possible bgl^+ mutants were minimal agar containing: 0.075% yeast extract (Difco),

0.5% salicin and 0.02% bromothymol blue (Schaefler 1967).

2. III. GENETIC PROCEDURES

a) Selection of lacy$^+$ revertants.

In order to reduce background growth due to the leakiness of the lacy$^-$ mutation, a culture of P162-8 growing exponentially in minimal medium supplemented with 40µgm/ml of thymine and the amino acid requirements, and containing 1mMlactose was diluted 10^2 fold and spread onto minimal plates containing 0.5mM lactose.

b) 2-aminopurine mutagenesis and penicillin enrichment.

10 ml of nutrient broth supplemented with thymine (40µgm/ml) and containing 2-aminopurine at 500µgm/ml was inoculated with MC5. Growth was allowed to continue for 6hrs at 37°C. The penicillin enrichment procedure used was essentially that of Gorini and Kauffman (1960). The culture was washed and resuspended in minimal medium lacking proline but containing thymine, threonine,leucine and thiamine. Growth was allowed to continue for 30 minutes at 37°C. Penicillin was then added at 200µgm/ml and the culture was grown for a further 3 hours. The cells were plated on non-selective minimal plates and subsequently replica-plated onto selective plates.

c) P1 transduction procedure.

The transduction procedure used was as follows: the donor strain was grown in 10ml of nutrient broth with bubbling aeration at 37°C

to an optical density at 450nm of about 0.7. 0.05ml of a 0.5M $CaCl_2$ solution was then added and aeration was continued for a further 10 minutes. The phage was added to 0.3ml of the donor to give a final titer of 10^7 plaque forming units per plate. The mixture was incubated at 37°C for 20 minutes to allow adsorption of the phage. Soft agar was added to the tubes, which were then poured onto Z-agar plates and incubated overnight. The transducing phage were isolated by removing the layer of soft agar (usually from 3 plates) into a sterile glass centrifuge tube. 0.3ml of chloroform and 1ml of T_2 adsorption medium were added to the tube. The mixture was then shaken vigorously, incubated for 15mins at 37°C and centrifuged for 5 mins in an MSE bench centrifuge. The supernatant (approximately 10ml) was mixed with 1ml of chloroform. The upper aqueous layer was removed and centrifuged again to remove cell debris. The resulting transducing preparation was stored at 4°C.

The recipient was grown in 10ml of nutrient broth to an optical density at 450nm of 0.7, centrifuged and resuspended in buffer containing 50mM $CaCl_2$. Phage were added at a multiplicity of infection of about 10 and the mixture was left at room temperature for 90 minutes. It was then centrifuged and resuspended in buffer containing sodium citrate at 0.5% to a concentration of about 10^9 cells/ml. The cells were spread onto selective plates.

d) Mating procedure.

Donor and recipient were grown to a density of about 2×10^8 cells/ml at 37°C. 1ml of each were mixed and incubated without agitation for 45 mins. In the case of the Flac matings, since the recipients were lac⁻, the cells were plated directly onto lactose minimal plates. The resulting colonies were purified and restreaked onto lactose tetrazolium plates. Only those colonies which exhibited some segregation were used. In the case of the Ftrp(KLF23) matings, no direct selection procedure could be used. After mating, therefore, the cells were plated onto minimal plates. The resulting recipient colonies were purified, patched onto minimal plates and replica plated onto nutrient agar plates. These were then replica plated onto EMBO plates supplemented with glucose and each spread with phage μ or MS2 (approximately 10^{10} per plate). Under these conditions, male clones are partially lysed and appear dark, whereas female clones appear pink.

e) Selection of β-glucoside utilizing mutants.

Strains of E.coli K_{12} cannot normally utilize β-glucosides (bgl⁻). Mutants which are able to utilize these sugars can be readily isolated by plating the cells on minimal plates containing arbutin and salicin.

Cultures growing exponentially in minimal medium were spread onto Aarb.sal. plates. After two days, the resulting colonies were purified and tested on ABsal. plates. bgl⁻ colonies are white,

bgl$^+$ colonies are yellow on these plates.

f) Screening procedure for tna$^-$ transductants.

Purified clones were grown overnight in tryptone broth containing thymine (40 µgm/ml) and tryptophan (20 µgm/ml).0.2ml of Kovacs reagent (5gm of p-dimethylaminobenzaldehyde in 75 ml of amyl alcohol and 25 ml of concentrated HCl) was added to 1 ml of culture. tna$^+$ clones give a characteristic red colouration to the organic phase. tna$^-$ clones appear yellow. (Difco Manual Ninth Edition 1971).

g) Selection procedure for trpR$^-$ transductants of the tna$^-$ strain.

The recipient cells were plated directly onto selective plates containing 100 µgm/ml 5-methyltrophan and incubated for 2-3 days. Several large colonies were isolated and purified (Cohen and Jacob 1959).

These isolates were screened in the following manner: cultures were grown overnight in glycerol minimal medium containing the required supplements, in the presence of tryptophan (100-200 µgm/ml). The cultures were adjusted to the same optical density (OD_{450}) and an equal volume of each was centrifuged. The pellet was resuspended in 1ml of phosphate buffer and 4ml of substrate (containing 2.0 moles indole,400 µmoles DL-serine,500 µmoles Tris-chloride buffer (pH-7.8), 100 µgm pyridoxal phosphate, 0.15 ml saturated NaCl and distilled water to a final volume 4 ml) (Ito and Crawford 1965) was added.

After 15 minutes, 1ml was removed into 0.1ml of 1M NaOH. 1 ml of indole reagent (Smith and Yanofsky 1962) was then added. $\underline{trpR}^+$ strains give a red colouration whereas $\underline{trpR}^-$ strains are yellow.

2. IV. BACTERIAL GROWTH AND MEASUREMENT OF GROWTH PARAMETERS.

a) Optical density.

The optical density of the cultures was monitored at a wavelength of 450nm (OD_{450}) with a Gilford microsample spectrophotometer.

b) Cell number.

Cell number was determined using a Coulter electronic particle counter model B, with a 30 μ orifice. Samples of the culture were mixed with an equal volume of 0.4% formaldehyde in isotonic saline.

c) Relative amounts of DNA.

Samples of the culture grown in the presence of ^{14}C-thymine (Specific activity of 0.05 μCi/μgm) were mixed with an equal volume of 10% TCA (trichloroacetic acid) at 4°C. They were left for at least 30 minutes before being filtered through a membrane filter (Sartorius 0.45 μm pore size, 27mm diameter). The filtered samples were washed with 5 x 10 ml volumes of hot (95°) distilled water and were dried using infra-red lamps. They were placed with a constant orientation in small ($\frac{1}{2}$" x 1$\frac{1}{2}$") glass vials. 3.5ml of non-aqueous scintillation

fluid was added to each vial (PPO 5gms,POPOP 0.3gm in 1 liter of toluene) (Pritchard and Lark 1964). They were placed in standard glass scintillation vials and counted in a packard liquid scintillation counter.

d) Bacterial growth.

Medium was inoculated with cells from a minimal agar plate and the culture aerated vigorously at 37°C in a New Brunswick gyrotory shaking water bath.

The optical density of the culture was never allowed to exceed 0.3 during the course of an experiment. In general, the optical density of the culture was kept between 0.1-0.2 (and occasionally between 0.05-0.1) by 2 fold dilution into fresh, prewarmed medium containing the required concentration of thymine. It is thus a simple matter to decrease the concentration of thymine in the growth medium (a step-down) by decreasing the concentration of thymine in the dilution medium, and vice versa (a step-up). Samples were removed periodically for optical density measurements, and either DNA measurements or enzyme assays.

2. V. CONDITIONS FOR THE ENZYME ASSAYS

a) β-Galactosidase.

The procedure used for estimating the potential of the cells to produce β-galactosidase was as follows: 1ml samples were removed from

the culture and induced for a short period of time (in most cases, 10 minutes at 37°C) with isopropylthiogalactoside (IPTG) at a final concentration of 5×10^{-4}M. The enzyme was assayed according to the method of Pardee et.al. (1959). The cells were killed by the addition of a 1/20th volume of toluene and incubated with shaking at 37°C for 10 minutes. The samples were then brought to 28°C and 0.2ml of a M/75 solution of o-nitrophenolgalactoside (ONPG) in 0.25M phosphate buffer (pH=7.0) added. Incubation was continued for 15-20 minutes. The reaction was stopped by the addition of 0.5 ml of 1M Na_2CO_3 and the optical density at 420nm was read after centrifuging the samples to remove any unlysed cells.

Under these conditions, the reaction of the enzyme with substrate in linear for at least 40 minutes (figure 6), and over the range of culture mass (OD_{450}) used, the enzyme activity shows good proportionality (figure 7).

Measurement of basal levels was carried out in the same manner with the omission of the induction step. The reaction in this case was allowed to continue overnight at 28°C.

One unit of enzyme activity is defined as producing 1m μ-mole of o-nitrophenol per minute at 28°C.

b) Tryptophanase.

The assay procedure used was that of Pardee and Prestidge

FIGURE 6.

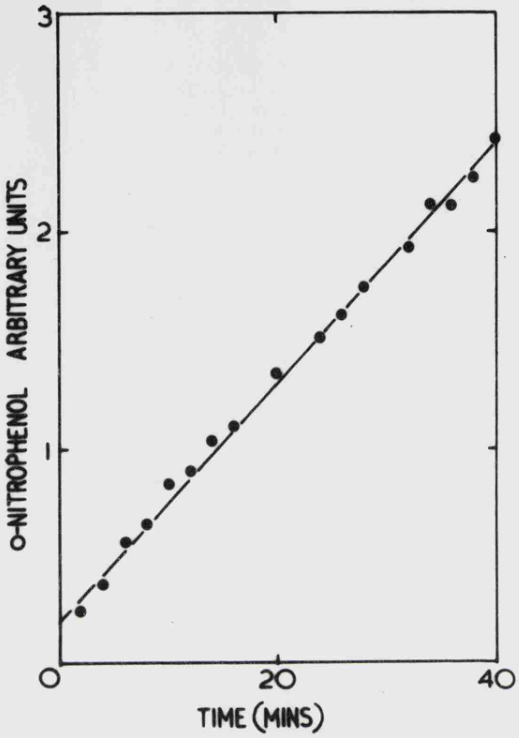
REACTION OF β-GALACTOSIDASE WITH SUBSTRATE.

MC11 was grown in the presence of 5×10^{-4} M IPTG to an optical density at 450 nm of 0.2. The assay procedure is described in Material and Methods. The blank has not been subtracted.

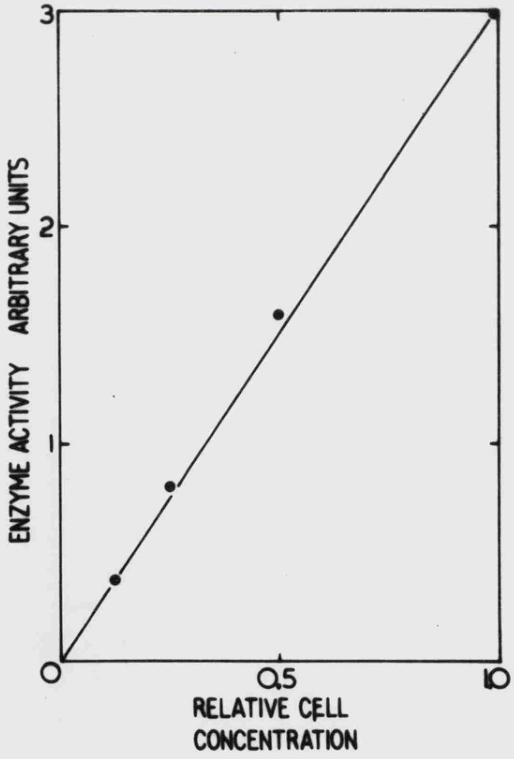
FIGURE 7.

PROPORTIONALITY BETWEEN β-GALACTOSIDASE ACTIVITY AND CELL CONCENTRATION.

MC11 was grown as above to an OD_{450} of 0.3, killed with toluene and incubated at 37°C for 10 minutes. The culture was then diluted 1 in 2, 1 in 4 and 1 in 8.



REACTION OF β GALACTOSIDASE WITH SUBSTRATE



PROPORTIONALITY BETWEEN ENZYME ACTIVITY AND CELL CONCENTRATION

(1961), and was as follows: 1ml samples of culture were induced (usually for 20 minutes) by the addition of L-tryptophan (final concentration of 0.5mg/ml).0.2ml of the induced culture was killed by the addition of toluene, as for β-galactosidase, and 1ml of substrate was added (containing per milliliter, 75 μgm of pyridoxal phosphate and 2.5 mg of L-tryptophan). The mixture was incubated at 37°C for 60 minutes.0.9ml of Ehrlichs reagent was then added (5 parts of a solution of 2.0gm of p-dimethylaminobenzaldehyde in 80ml of 99% ethanol to 12 parts of a mixture of 16ml concentrated H_2SO_4 in 200 ml of 99% ethanol) and the optical density was read after 20 minutes at 568nm. The optical density readings were multiplied by 0.027 to give μ-moles of indole released (Pardee and Prestidge 1961).

Under these conditions, the reaction of enzyme with substrate is linear (figure 8), and the enzyme activity is proportional to the optical density of the culture at 450nm. (figure 9).

One unit of enzyme is defined as the amount of enzyme that causes the formation of 1 μ-mole of indole per minute.

c) <u>D-serine deaminase.</u>

The assay procedure used was that of Pardee and Prestidge (1955). A sample of culture was induced by the addition of D-serine (150 μgm/ml) for 30 minutes. Induction was terminated by treating the cells with toluene.0.2ml of the induced culture was mixed with

FIGURE 8.

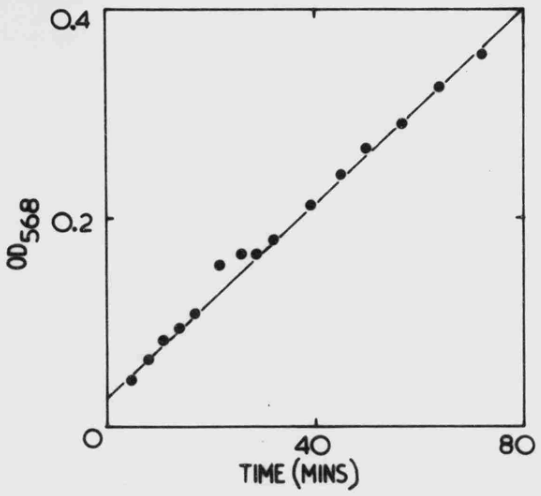
REACTION OF TRYPTOPHANASE WITH SUBSTRATE.

Mc11 was grown to an OD_{450} of 0.2 and induced for 20 minutes by the addition of L-tryptophan (0.5 mg/ml). The enzyme assay procedure is described in Materials and Methods. The blank has not been subtracted.

FIGURE 9.

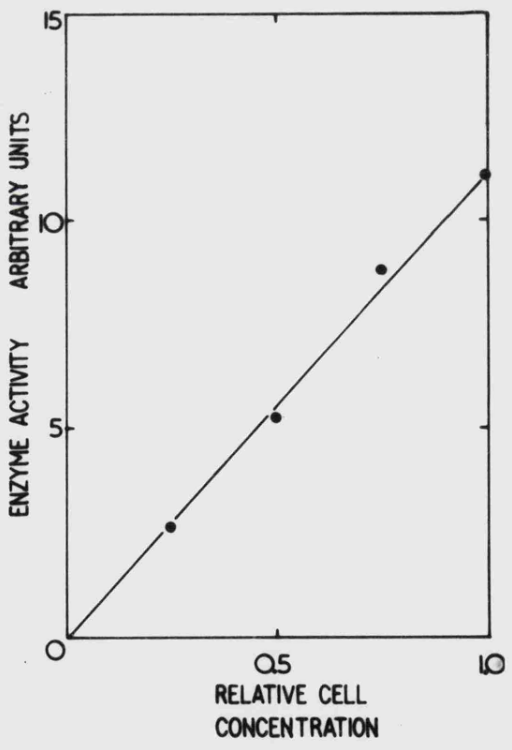
PROPORTIONALITY BETWEEN TRYPTOPHANASE ACTIVITY AND CELL CONCENTRATION.

MC11 was grown as above to an OD_{450} of 0.3 and induced for 20 minutes, diluted and assayed as described in Materials and Methods.



REACTION OF TRYPTOPHANASE WITH SUBSTRATE

PROPORTIONALITY BETWEEN ENZYME ACTIVITY AND CELL CONCENTRATION



0.2ml of substrate (0.04M D-serine,0.01M sodium formate and 0.4mg/ml EDTA in 0.04M phosphate buffer, pH 8.0) and incubation continued at 37°C for 15 minutes.0.9ml of a solution of 2,4-dinitrophenylhydrazine (0.17mg in 1ml of 1.2N HCl) was added and the mixture incubated for 20 minutes. The mixture was incubated for a further 10 minutes after the addition of 1.7ml of 2.5N NaOH and the optical density read at 520nm.

Under these conditions, the reaction of enzyme with substrate is linear (figure 10), and the enzyme activity is proportional to the optical density of the culture at 450nm. (figure 11).

d) Tryptophan synthetase.

Tryptophan synthetase was assayed essentially according to Lester and Yanofsky (1960).25ml samples were filtered onto a membrane filter (Sartorius 0.45 μpore size), washed with prewarmed buffer (0.05M phosphate,pH7.8) and resuspended in 1ml of substrate containing 0.4 μ-moles of indole,6.3mg of D,L-serine and 10 μgm of pyridoxal phosphate. The reaction mixture was incubated at 37°C and the reaction terminated by the addition of 0.1ml of 1N NaOH followed by 4ml of toluene. Samples of the toluene extract were assayed for the disappearance of indole in the following way (Smith and Yanofsky 1962): 1.0ml samples of the toluene extract were added to 4ml of 95% ethanol and 2ml of indole reagent (9gm of p-dimethylaminobenzaldehyde dissolved in 200ml of 95% ethanol and made up to 250ml with 45ml of concentrated HCl and 95% ethanol). The mixture

FIGURE 10.

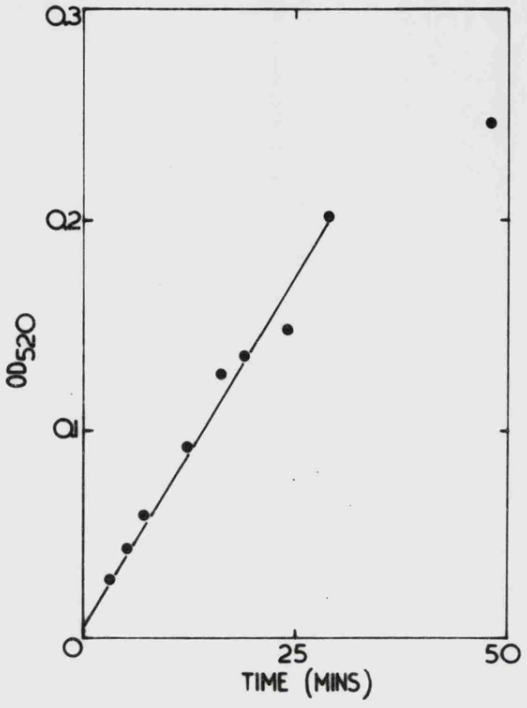
REACTION OF D-SERINE DEAMINASE WITH SUBSTRATE.

MC11 was grown to an OD_{450} of 0.2 and induced for 30 minutes by the addition of D-serine (150 ugm/ml). The assay conditions are described in Materials and Methods.

FIGURE 11.

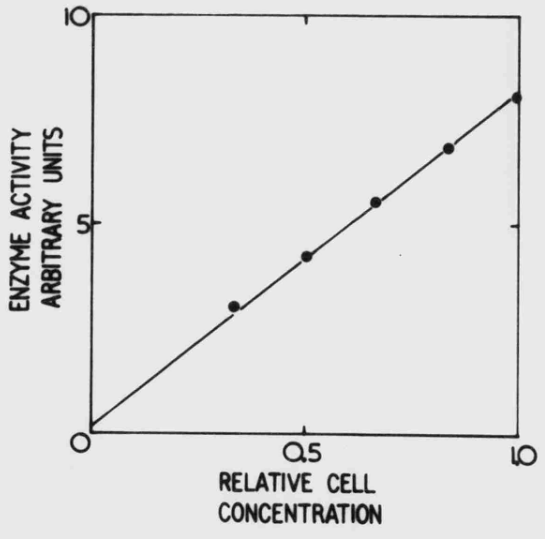
PROPORTIONALITY OF D-SERINE DEAMINASE ACTIVITY WITH CELL CONCENTRATION.

MC11 was grown to an OD_{450} of 0.3 induced for 30 minutes, diluted and assayed as described in Materials and Methods.



REACTION OF D-SERINE DEAMINASE WITH SUBSTRATE

PROPORTIONALITY BETWEEN ENZYME ACTIVITY AND CELL CONCENTRATION



was shaken and left for 20 mins at room temperature. Its optical density was then read at 540nm.

Values in the text are expressed in relation to the culture mass (OD_{450}) before concentration. However, since the percentage yield after resuspension was always the same and did not vary with the initial concentration of cells, the error introduced in doing this was negligible.

A typical reaction profile (figure 12) shows that under these conditions, the reaction of enzyme with substrate is linear.

e) Thymidine phosphorylase.

This enzyme was assayed according to Beacham et.al.(1968). as follows: 25ml samples were filtered onto membrane filters (Sartorius 0.45 μpore size) and resuspended in 10ml of arsenate buffer (50mM arsenate, pH 6.0). The cells were then killed by toluene treatment. For the assay, an equal volume of substrate (20mM thymidine in arsenate buffer) was added to the suspension at 37°C and 1ml samples were removed periodically into 1.0ml 0.2N NaOH. After 30 minutes, the thymine released was measured at 300nm. Values in the text are again expressed in relation to the culture mass before concentration.

The reaction of the enzyme with substrate was linear over the period of the assay as can be seen from a typical assay (figure 13). The enzyme activity was also proportional to OD_{450} (figure 14).

FIGURE 12.

REACTION OF TRYPTOPHAN SYNTHETASE WITH SUBSTRATE.

MC32 was grown to an OD_{450} of 0.3. 200 ml of culture was centrifuged and resuspended in 10 ml of substrate. The assay is described in Materials and Methods.

REACTION OF TRYPTOPHAN SYNTHETASE
WITH SUBSTRATE

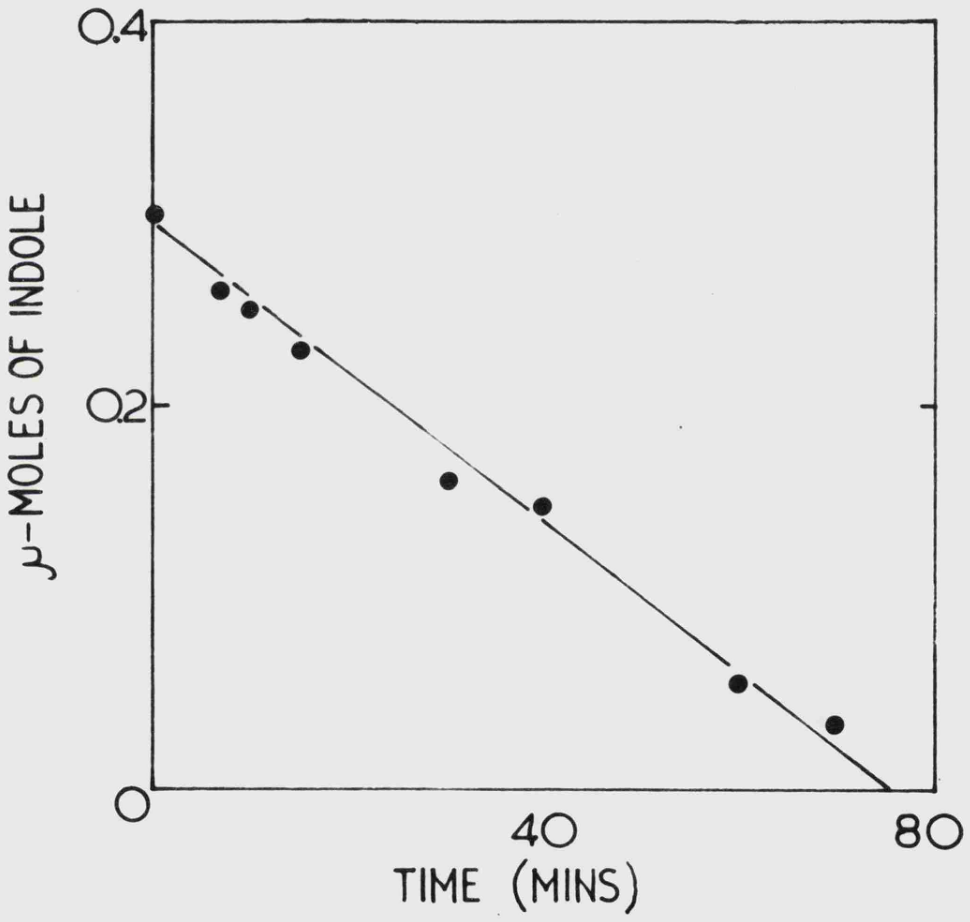


FIGURE 13.

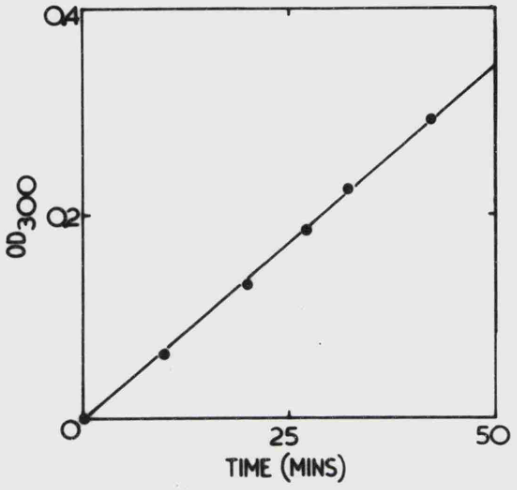
REACTION OF THYMIDINE PHOSPHORYLASE WITH SUBSTRATE.

MC17 was grown to an OD_{450} of 0.2.25 ml of cells were filtered and resuspended in 10 ml. of arsenate buffer (50 mM arsenate pH 6.0). An equal volume of substrate was added at 37°C. Samples were removed at intervals and assayed as described in Materials and Methods.

FIGURE 14.

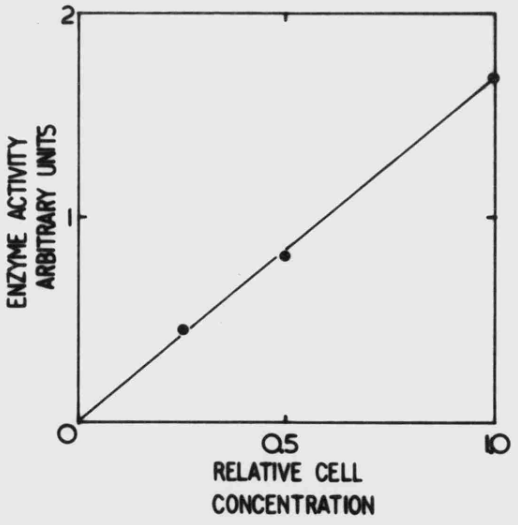
PROPORTIONALITY BETWEEN THYMIDINE PHOSPHORYLASE ACTIVITY AND CELL CONCENTRATION.

MC17 was grown to an OD_{450} of 0.3.100 ml. of culture was filtered, washed and resuspended in 0.5ml. of 50 mM. arsenate buffer pH 6.0. Following incubation with 1/20th volume of toluene, the culture was diluted and assayed as described in Materials and Methods.



REACTION OF THYMIDINE PHOSPHORYLASE WITH SUBSTRATE

PROPORTIONALITY BETWEEN ENZYME ACTIVITY AND CELL CONCENTRATION



3. GENE CONCENTRATION, RELATIVE GENE DOSAGE AND GENE OUTPUT

3. I. BALANCED AND STEADY-STATE GROWTH

Balanced growth was defined by Campbell (1957) as a parallel increase in all extensive parameters of the growing system. Thus in a culture growing in a balanced exponential manner, cell mass, DNA,RNA and cell number all increase at the same rate. It has been shown however, (Zaritsky and Pritchard 1973) that thymine low requiring strains of E.coli under certain conditions, never reach a balanced state of growth. Under conditions in which the transit time (C) is much longer than the doubling time (τ), the mass doubling time is less than the doubling time of cell number. The cells therefore increase in size continuously. Under these circumstances the DNA:mass ratio of the culture remains constant. It therefore seems as though cell division has been uncoupled from cell growth. This phenomenon is at present poorly understood. Although under these conditions, the culture is not balanced, it is in a steady state of growth. This observation, as will be shown later, enables gene dosage (as defined here) to be ruled out as an important factor in determining gene output.

The criteria which were used to define "normal" growth (Zaritsky and Pritchard 1971) are:-

I) an exponential increase in cell mass which is independant of the thymine concentration in the growth medium.

and 2) a constant DNA:mass ratio.

a) thymine requirement of the strains used in these studies

In order to discover the lowest concentration of thymine on which the cells would grow, overnight cultures of several strains were grown with limiting glucose in 5μgm/ml of thymine. These were then diluted 10^2 fold and 0.I ml of each spread onto minimal glucose plates containing various concentrations of thymine. The results are shown in table I. P-1628 was the original strain used by Zaritsky and Pritchard (1971) and was found, in agreement with their results, to be unable to grow on I μgm/ml of thymine. This level of thymine however, supported growth of the parental strain from which the derivatives used in this study were made (although this parental strain was also thought to be P-I628). The reason for this difference in thymine requirement is not clear. It is possible that the parental strain used here was a so-called "super-low" thymine requiring strain (Ahmad and Pritchard 1970) but this is unlikely for two reasons. In the first place, most super-low requirers are constitutive for the enzyme thymidine phosphorylase, and the strains used in these studies produce this enzyme at a basal rate. In the second place, these strains will not grow on glucose minimal plates containing less than I μgm/ml of thymine, whereas super-low requirers will grow on at least half this concentration under these conditions (Ahmad and Pritchard 1970). A second possibility is that the thy⁻ mutation has become leaky. I have not tested this possibility since the results presented in this thesis will not be affected by the leakiness of the thy⁻ mutation.

TABLE 1 THE THYMINE REQUIREMENT OF THE STRAINS USED IN THESE STUDIES

strain	[thy] µgm/ml	5	2	1	0.5	0.25	0.1
			VIABLE COUNT				
P162-8		270	200	3	-	-	-
MC9		332	370	332	56	-	-
MC17		270	320	280	88	-	-

The experimental procedures used are described in the text.

b) steady-state growth in batch culture : DNA:mass ratio

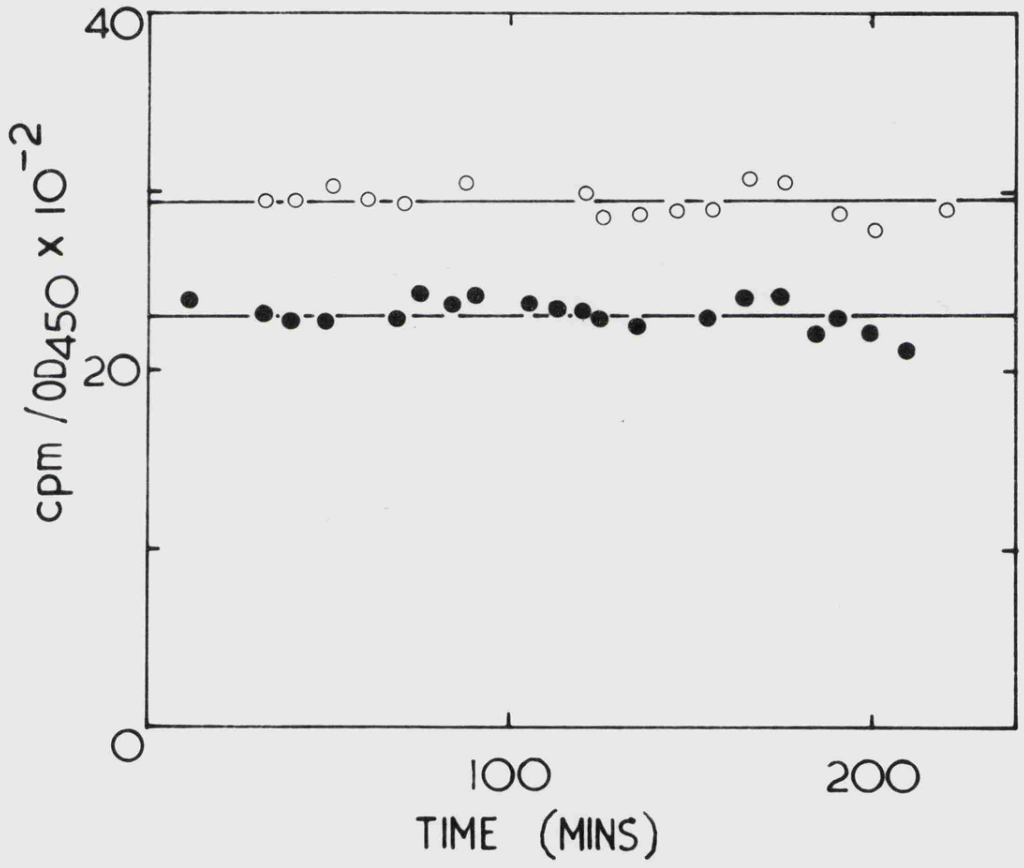
In batch culture the original parental strain and its derivatives grow normally in I μgm/ml of thymine for at least 5 generations either in glycerol-cas medium (supporting a mass doubling time of 30 minutes) or in glycerol amino acid medium (supporting a mass doubling time of 55 minutes). The DNA:mass ratio ($\bar{G}/\bar{M}$) of such a culture over 4 generations, growing with a mass doubling time of 55 minutes is shown in figure 15. This ratio remains constant over the course of the experiment. Also shown in figure 15 is the DNA:mass ratio of the same strain growing in 40 μgm/ml of thymine. The observed increase in $\bar{G}/\bar{M}$ has been shown to reflect an increase in the replication velocity (a decrease in C) in high thymine (Pritchard and Zaritsky 1970).

The change in the transit time (ΔC) was calculated according to Pritchard and Zaritsky (1970). Changes in $\bar{G}/\bar{M}$ give only relative values of C.To evaluate the absolute values it is necessary to define a set of growth conditions (that is, growth rate and thymine concentration) under which the absolute replication velocity is known. In practice however, this absolute value is not critical in determining the magnitude of ΔC, and a range of values may be used without significantly affecting the result. In general, the value of C, for purposes of calculation only, was assumed to be 55 minutes at high thymine concentrations (40 μgm/ml) (Zaritsky and Pritchard 1971). The values of C at lower thymine concentrations were calculated from this

FIGURE 15.

DNA:MASS RATIO IN HIGH AND LOW THYMINE

Values are expressed in cpm/ml/OD_{450} x 10^{-2}; (o) 40 µgm/ml.thymine; (•) 1 µgm/ml.thymine. The cells were grown as described in Materials and Methods, and had a mass doubling time of 55 minutes. In this experiment, the culture did not undergo a step-up, instead measurements were made on two separate steady-state batch cultures.


40
20
0
cpm /OD450 x 10-2
100
200
TIME (MINS)

reference value and the observed changes in $\bar{G}/\bar{M}$. The values of $\bar{G}/\bar{M}$ for the three strains MC11, MC17 and MC32 in 1 and 40 μgm/ml of thymine with identical specific activities of ^{14}C-thymine and growing under the same cultural conditions, were identical. The calculated change in C for a 1-40 μgm thymine step was 36 minutes.

c) protein:mass ratio.

Although unlikely, it could be argued that the predicted changes in gene concentration and relative gene dosage might result in a change in the differential rate of total protein synthesis and thus lead to a change in the protein:mass ratio of the culture. It is shown in Figure 16 that this is not the case. A culture, fully labeled with ^{14}C-L-histidine (specific activity of 0.0013 μCi/μgm) and growing in either 1 or 40 μgm of thymine per ml, was sampled in the same manner as was used in the determination of the DNA:mass ratios (see section 2.IV). There is no detectable difference between the 1 and 40 ugm cultures under these conditions. The implication here is that the specific rate (rate per gene) of protein synthesis must fall as the DNA concentration increases.

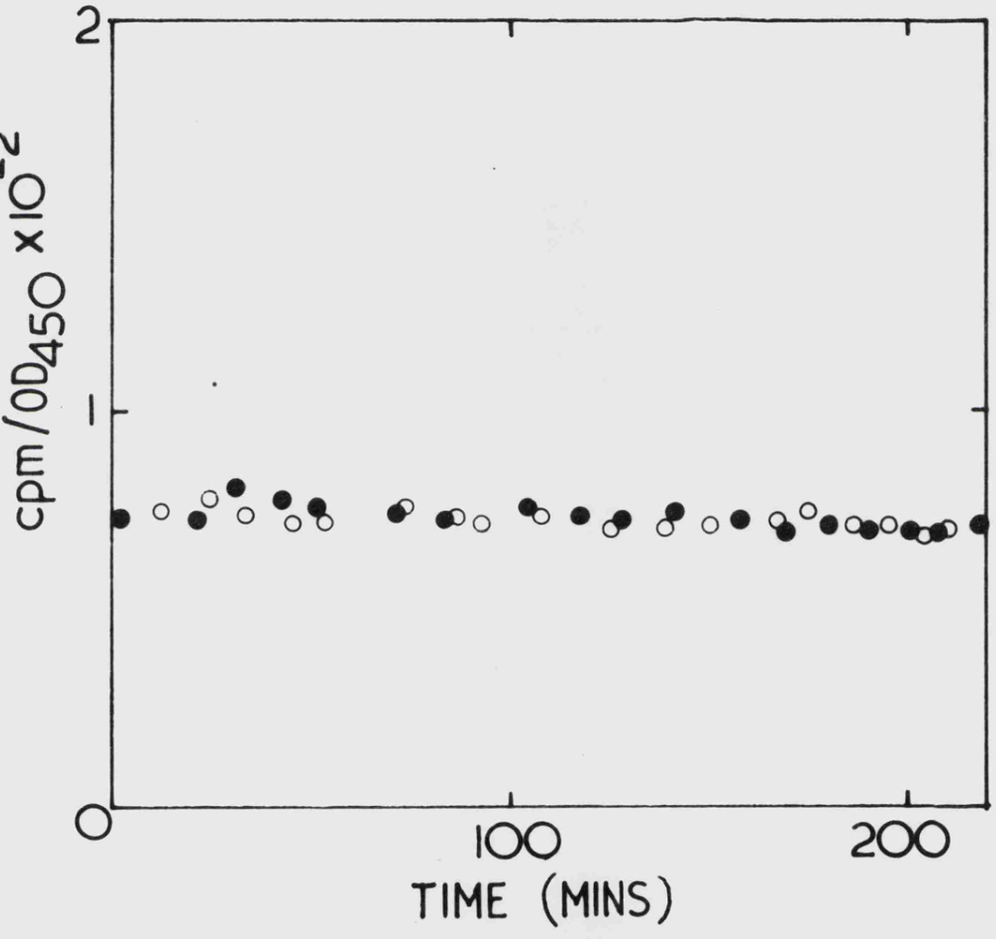
d) change in cell size.

The model proposed by Cooper and Helmstetter (1968) for the bacterial cell cycle leads to the prediction that the average cell size ($\bar{M}$ - equation 6) will increase with decreasing values of C. This prediction has been verified by Zaritsky and Pritchard (1973).

FIGURE 16.

PROTEIN:MASS RATIO IN HIGH AND LOW THYMINE

The experimental procedures are described in Materials and Methods. The values are expressed as cpm/ml/OD_{450} x 10^{-2}. Experimental conditions were as in figure 15; (o) 40 µgm/ml. thymine; (•) 1 µgm/ml. thymine.


2
1
0
cpm/OD450 x10-2
100
200
TIME (MINS)

However, as I have already stated, these authors have shown that under certain conditions, cell size increases continuously. I have also observed this phenomenon in MC17 and in an F'lac derivative, MC10 (figure 17 a and b). The increase in cell size is quite marked when the cells are grown in low thymine (1 μgm/ml) and almost undetectable when grown in high thymine (40 μgm/ml) in the glycerol amino acid medium with a mass doubling time of 55 minutes.

Since the DNA concentration ($\bar{G}/\bar{M}$) remains constant in low thymine (figure 15), this increase in cell size has no effect on gene concentration or relative gene dosage because the former is related to mass and the latter to total DNA. It does however, have some bearing on gene dosage ($\bar{F}$) as it has been defined earlier. The observed increase in cell size ($\bar{M}$) must be accompanied by a similar increase in $\bar{G}$ in order that $\bar{G}/\bar{M}$ remains constant. The dosage of any gene, irrespective of its position relative to the origin must also increase at the same rate.

3. II. THE EFFECT OF THYMINE CONCENTRATION ON THE OUTPUT OF β-GALACTOSIDASE.

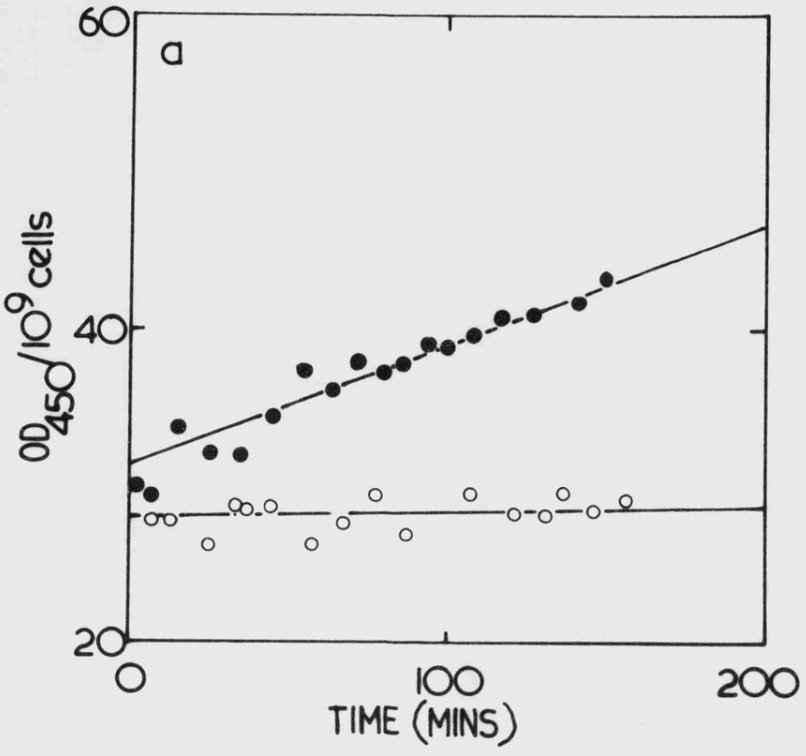
The gene specifying β-galactosidase is probably located some distance from the chromosome origin in E.coli (Masters and Broda 1971; Bird et.al. 1972). It would therefore be anticipated that a change in replication velocity would lead to a substantial change in its

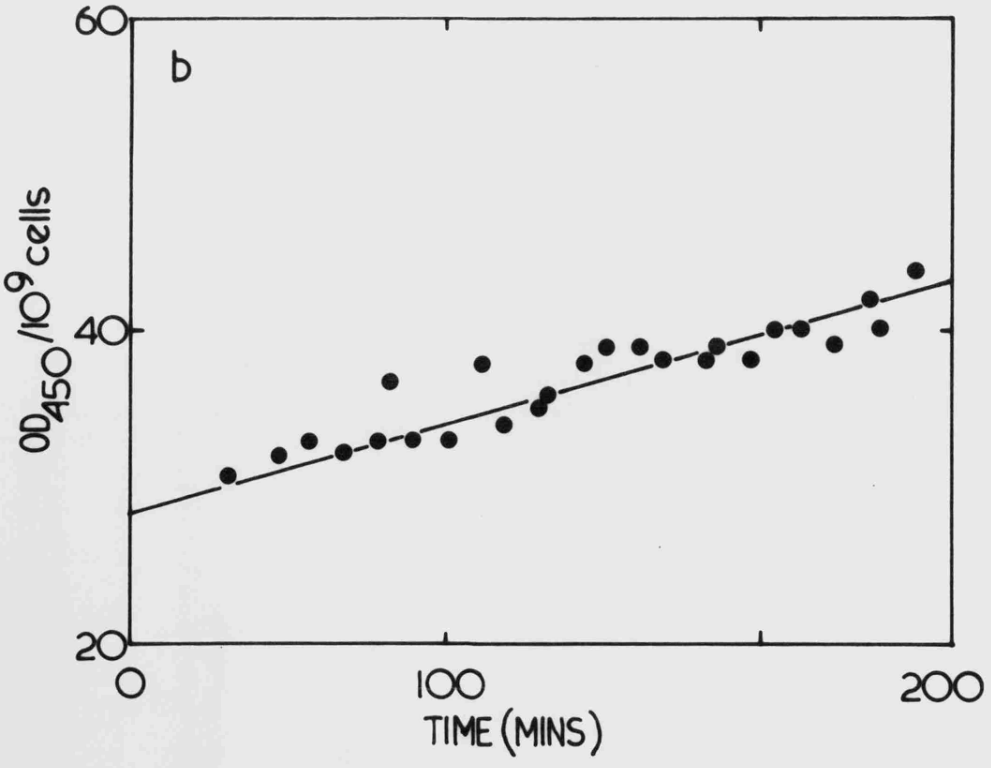
FIGURE 17.

CHANGE IN CELL SIZE WITH TIME.

a The change in cell size of MC17 growing with a mass doubling time of 55 minutes in (•) 1 μgm/ml.thymine and (o) 40 μgm/ml.thymine.

b The change in cell size of MC10 an Flac derivative, growing as above in 1 μgm/ml.thymine.


a
60
40
20
0
100
200
TIME (MINS)
$OD_{450}/10^9$ cells


b
60
40
20
0
100
200
TIME (MINS)
$OD_{450}/10^9$ cells

concentration and might also lead to a change in its relative dosage.

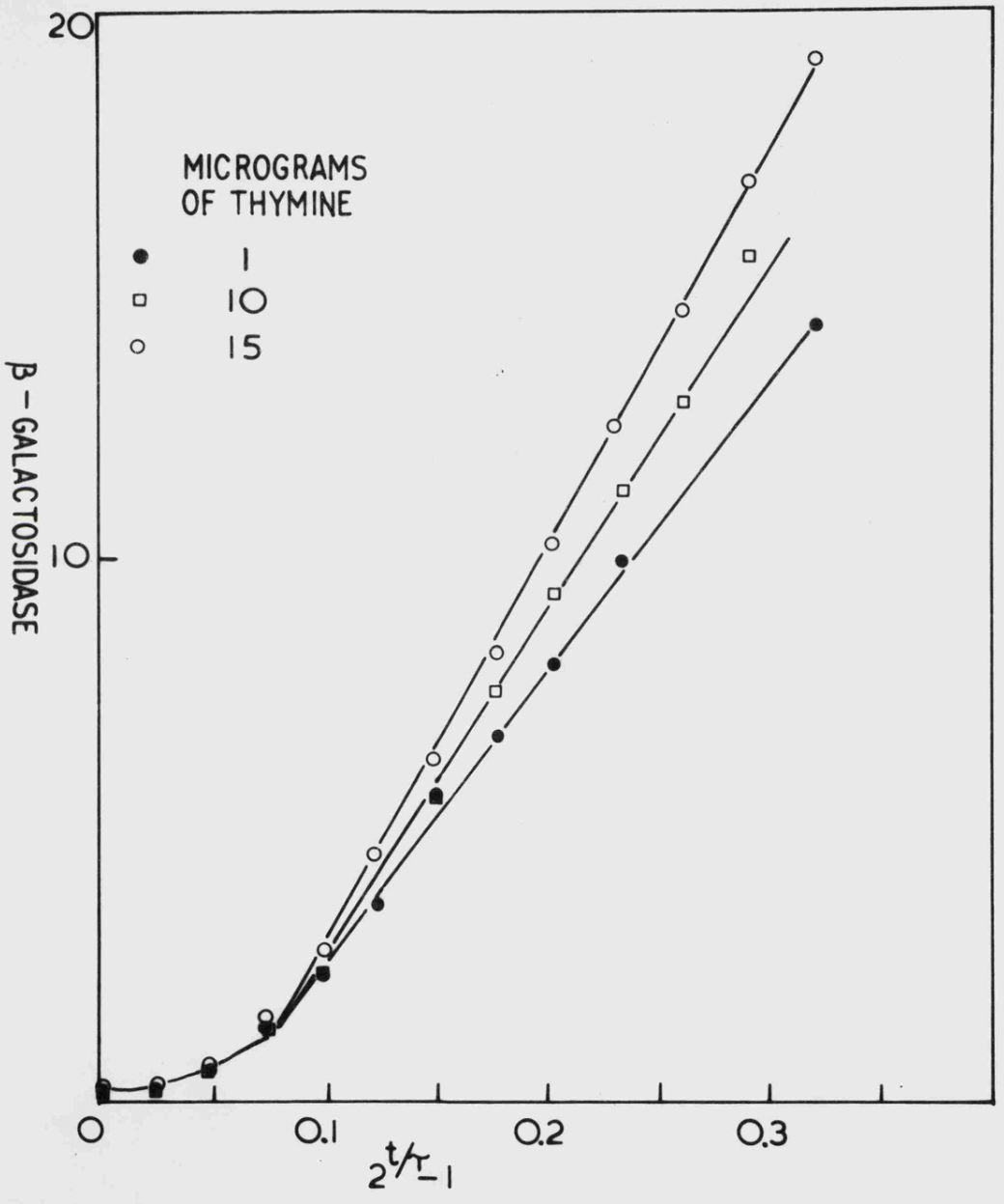
The first experiment was therefore to determine whether a change in thymine concentration in the growth medium of the thy⁻ strain leads to a change in the differential rate of synthesis of β-galactosidase. The results of such an experiment are shown in figure 18. The scale on the abscissa represents the increase in culture mass. At $t = 0, 2^{t/\tau} - I = 0$, at $t = \tau$, $2^{t/\tau} - 1 = 1$ and 1 unit of mass has been made. β-galactosidase units have been normalised to the culture mass at t = 0 (time of addition of inducer). It can be seen that, as expected, the higher the concentration of thymine, the higher was the differential rate of synthesis of β-galactosidase, although the growth rate (rate of increase in absorbance) was identical in each growth medium (data not shown).

A more instructive way of demonstrating this effect is to perform an experiment in which the culture is "stepped-up" (Pritchard and Zaritsky 1970) from one thymine concentration to a higher one and the transition from one steady-state differential rate of synthesis to another determined. Such an experiment is analogous to the well known "shift-up" experiments of Kjeldgaard et.al. (1958), except that instead of following a transition from one growth rate to another, the transition from one replication velocity to another is followed. The

FIGURE 18.

EFFECT OF THYMINE CONCENTRATION ON THE DIFFERENTIAL RATE OF β-GALACTOSIDASE SYNTHESIS.

The cells were grown in glycerol Casamino acid medium containing 1,(●); 10,(□) and 15,(o) μgm/ml.thymine. Inducer was added at t = 0. The scale on the abscissa is a measure of the mass increase of the culture. The curves are thus a direct measure of the differential rate of synthesis of β-galactosidase. The levels of β-galactosidase were corrected for differences in the culture mass at t = 0 in these experiments.


20
10
0
0.1
0.2
0.3
MICROGRAMS
OF THYMINE
1
10
15
β - GALACTOSIDASE
$2^{t/\tau}-1$

results of such an experiment are shown in figure 19. In this type of experiment, samples taken at successive times, from a culture growing under steady-state conditions on one thymine concentration were pulse induced with isopropylthiogalactoside (IPTG) and the enzyme synthesised during the pulse related to the culture mass at the time of addition of inducer. The concentration of thymine in the growth medium was then increased and measurement of the rate of enzyme synthesis following pulse induction was continued. Average determinations of the initial steady-state differential rate of synthesis (potential) were thus obtained in the two thymine concentrations, based on many determinations and the average change in output computed. This method provides firmer evidence in any one experiment that the culture was in a steady-state of growth with respect to the initial differential rate of β-galactosidase synthesis.

Estimation of the differential rate of enzyme synthesis in medium containing different concentrations of thymine by this technique is of course only valid because the initial induction kinetics (figure 18) are not affected by the thymine concentration.

It could be argued that the change in differential rate of synthesis of β-galactosidase found in experiments of the type shown in figures 18 and 19 is not directly related to the change in concentration or relative dosage of the β-galactosidase gene, but rather to changes in

FIGURE 19.

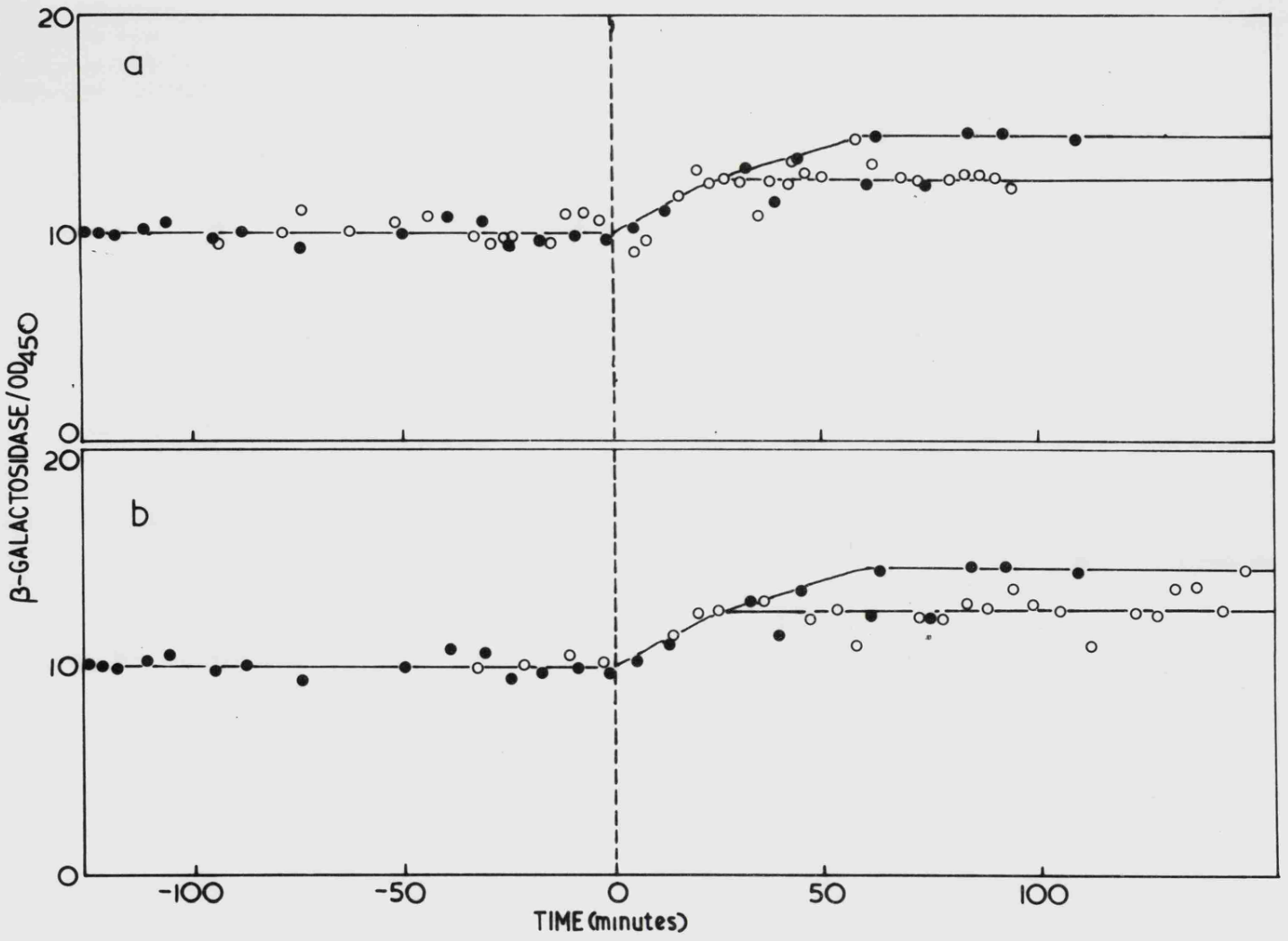
THE EFFECT OF THYMINE CONCENTRATION AND MASS DOUBLING TIME ON THE POTENTIAL FOR SYNTHESIS OF β-GALACTOSIDASE.

a The effect of thymine concentration.

MC5 was grown in low thymine (1 μgm/ml) glycerol Casamino acid medium and diluted at time zero into fresh, prewarmed medium with a final thymine concentration of 20 μgm, (•) or 10 μgm, (o). The mean values in low thymine have been normalised with respect to each other since the induction period used in each experiment was different.

b The effect of growth rate.

Cells were grown either in glycerol Casamino acid (•) or glycerol minimal medium supplemented with several amino acids (o) (see Materials and Methods) in the presence of 1 μgm/ml. thymine. At time zero, both cultures were diluted into medium containing thymine to give a final concentration of 20 μgm/ml. The mean values in low thymine were again normalised to each other.


20
a
10
0
20
b
10
0
β-GALACTOSIDASE/OD450
-100
-50
0
50
100
TIME (minutes)

cell physiology (e.g. changes in the degree of catabolite repression). This is unlikely, since changes in the output of β-galactosidase and other enzymes are quantitatively in agreement with independent estimates of the changes in gene concentration involved (see section 3.IV.). Moreover, no such step in β-galactosidase output was noted in a Δlac/F'lac derivative of this strain (see section 4.II).

Although in these studies I was not interested in the kinetics of the transition from one differential rate to another after a step-up, but only in the initial and final steady state values, it is of interest to note that if gene concentration determines gene output under these conditions, the time taken for the transition should, in a similar manner to the transition from one DNA:mass ratio to another, be a function of C_2, the transit time of a replication fork in the post step-up medium (Pritchard and Zaritsky 1970). In addition, it will also be a function of x, the distance of the gene from the origin of replication as a fraction of the total replicon ($0 \leqslant x \leqslant 1$). The time taken for the transition will be Cx minutes. This arises in the following way: An increase in replication velocity will result in an immediate increase in the frequency of termination of rounds of replication, whereas the frequency of initiation remains constant (if indeed initiation is governed by the rate of mass increase). There will thus be a progressive reduction in the number of replication forks

per chromosome until such time as the frequency of termination is reduced to the frequency of initiation. This time will correspond to the time taken for the "youngest" replication fork at the time of the step to terminate since only at this time will the new steady-state spacing between the forks be reached. The transition period will thus be equal to the new value of C. However, since replication of the chromosome is polarised and the change in concentration of a gene is dependant on its position on the chromosome, the closer the gene is situated to the origin, the sooner will the final steady-state concentration for this gene be reached. The upper limit occurs for genes situated at the terminus ($x = 1$), the lower limit, for genes at the origin ($x = 0$). The actual shape of the transition will of course depend on the age distribution of replication forks.

The transition period in figure 19 is of the correct order of magnitude. The observed transition period will be affected by the length of the induction pulse, the longer the induction pulse, the shorter will be the observed transition period. This is because the cells are undergoing the transition during the induction period. The anomolous difference in transition times during the 1-20 and 1-10 μgm thymine steps (figure 19a) might at least in part be attributed to the fact that different induction periods were used in the two experiments, but, a contributory factor is most certainly the large

degree of scatter occuring during this period.

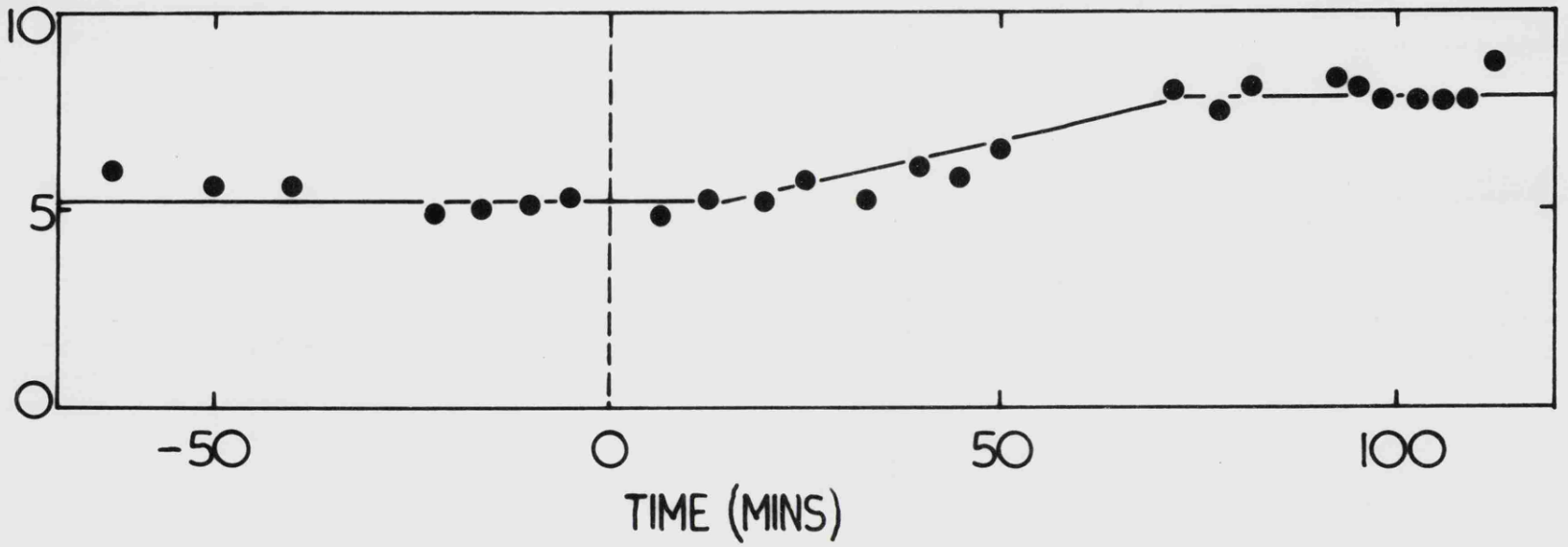
In relation to later experiments, it was important to determine whether the basal differential rate of synthesis of β-galactosidase responds quantitatively in an identical fashion to the induced rate. Figure 20 shows the results of an experiment in which the basal levels of β-galactosidase were measured under the same conditions as used in the experiments reported in figure 19 (●). There is good quantitative agreement between the basal and induced steps observed (1.44 for basal and 1.45 for induced levels in cells growing with a mass doubling time of 30 minutes and undergoing a 1 - 20 μgm thymine step). This shows that basal and induced synthesis are quantitatively related in an identical way with gene concentration or relative gene dosage. It should be noted here that the response of basal enzyme activity to changes in relative gene dosage or gene concentration should be much slower than the response of enzyme potential, reaching the final steady-state level asymtotically.

The equations describing the change in relative gene dosage and gene concentration (equations 8 and 12 and figure 3) predict an increase in the magnitude of this change for a given change in C with growth rate. The data shown in figure 19 are in accord with this prediction. The change in output during a 1 - 20 μgm step is greater for cells growing with a mass doubling time of 30 minutes than for those growing with a mass

FIGURE 20.

THE EFFECT OF THYMINE CONCENTRATION ON THE BASAL ACTIVITY OF β-GALACTOSIDASE.

Cells were grown in glycerol Casamino acid medium and stepped-up from 1 μgm/ml. to 20 μgm/ml.thymine. Values are expressed as enzyme activity (arbitrary units)/ml/OD_{450}. The substrate reaction was allowed to continue overnight at 28°C.


10
5
0
-50
0
50
100
TIME (MINS)

doubling time of 55 minutes.

3. III. CHANGES IN THE OUTPUT OF GENES AT OTHER CHROMOSOMAL LOCATIONS.

The qualitative results presented in the previous section allow no distinction to be made between the importance of gene concentration and relative gene dosage in affecting gene output. However, as can be seen from equations 8 and 12 and figure 3, the measurement of the change in output from a series of genes in cells growing at a fixed rate and undergoing a given change in replication velocity, should enable a distinction to be made.

I have measured the change in output of five genes during a step-up from low to high thymine. These were the genes specifying tryptophanase (at 73.5 minutes on the standard *E. coli* linkage map) (Taylor and Trotter 1972), thymidine phosphorylase (89.5), β-galactosidase (9), tryptophan synthetase (27) and D-serine deaminase (44.5).

As in the case of β-galactosidase, I measured the initial differential rate of synthesis of tryptophanase and D-serine deaminase following the addition of inducer. In the case of thymidine phosphorylase, I measured the basal differential rate of synthesis and in the case of tryptophan synthetase, a constitutive (*trp* R^-) strain was used.

It was necessary to measure the basal level of thymidine phosphorylase because the strain used in these studies was deoxyribomutase (drm$^-$) negative and thymidine phosphorylase is not inducible in such mutants (Barth et.al. 1968). In the case of tryptophan synthetase, the method commonly used to derepress this enzyme (viz: the addition of 3-methylanthranilic acid) would be inappropriate because such treatment might significantly change the growth rate and therefore interfere with the comparison I wished to make between the observed rates of enzyme synthesis and the predicted gene concentration or relative gene dosage under conditions of uniform growth rate.

Representative induction curves for tryptophanase and D-serine deaminase in cells growing in different thymine concentrations are shown in figure 21. Results for these enzymes from experiments similar to those shown in figure 19 for β-galactosidase are presented in figures 22 and 23.

The ratio of the enzyme levels in high thymine to those in low thymine for all five enzymes in cells undergoing a step-up from 1-40 μgm per ml of thymine are shown in figure 24 and table 2. Also included in table 2 are data from a similar series of experiments employing a 1 - 20 μgm thymine step. Figure 24 clearly shows that the effect of a given change in replication velocity on gene output depends on the position of the gene on the chromosome. The pattern obtained is qualitatively consistent with a bidirectional mode of

TABLE 2 THE EFFECT OF A CHANGE IN THYMINE CONCENTRATION ON GENE OUTPUT

a) 1-40 µgm thymine step

	LOCUS				
	lac	trp	dsd	tna	tpp
Ratio of enzyme activity	1.38 ± 0.04	1.40 ± 0.07	1.22 ± 0.06	1.02 ± 0.02	1.24 ± 0.05
Log ratio of enzyme activity	0.1383 ± 0.0115	0.1440 ± 0.023	0.0864 ± 0.0208	0.0085 ± 0.0095	0.0934 ± 0.0174

b) 1-20 µgm thymine step

	LOCUS		
	lac	dsd	tna
Ratio of enzyme activity	1.347 ± 0.04	1.197 ± 0.02	1.06 ± 0.03
Log ratio of enzyme activity	0.1294 ± 0.0131	0.0781 ± 0.006	0.0253 ± 0.0121

The change in gene output is expressed as the ratio of enzyme per unit mass in medium containing the higher thymine concentration to that in medium containing the lower concentration.

FIGURE 21.

THE EFFECT OF THYMINE CONCENTRATION ON THE DIFFERENTIAL RATE OF SYNTHESIS OF D-SERINE DEAMINASE AND TRYPTOPHANASE.

The cells were grown in glycerol amino acid medium (τ = 55 mins) in 1,(•) and 20,(o) μgm/ml.thymine. Inducer was added at t = 0.

a Tryptophanase.

b D-serine deaminase.

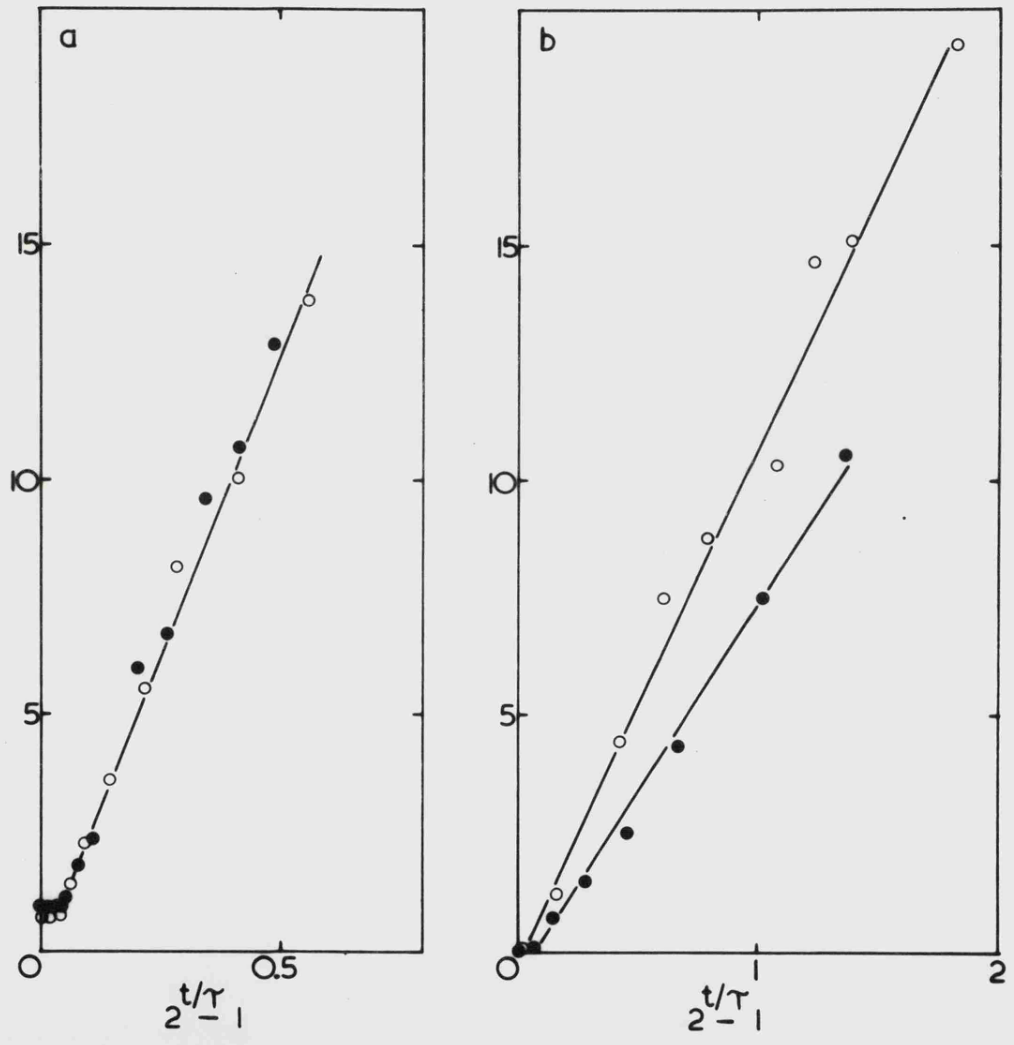

a
15
10
5
0
0.5
$2^{t/\tau} - 1$
b
15
10
5
0
1
2
$2^{t/\tau} - 1$

FIGURE 22.

THE EFFECT OF THYMINE CONCENTRATION ON THE SYNTHESIS OF TRYPTOPHANASE, D-SERINE DEAMINASE AND THYMIDINE PHOSPHORYLASE.

a Tryptophanase. (MC11)

b D-serine deaminase. (MC11)

c Thymidine phosphorylase. (MC17)

The assay conditions for these enzymes have been described in Materials and Methods. Steady-state cultures growing in 1 (•) or 40 (o) μgm/ml. thymine were employed rather than following the enzyme levels during a "step-up".

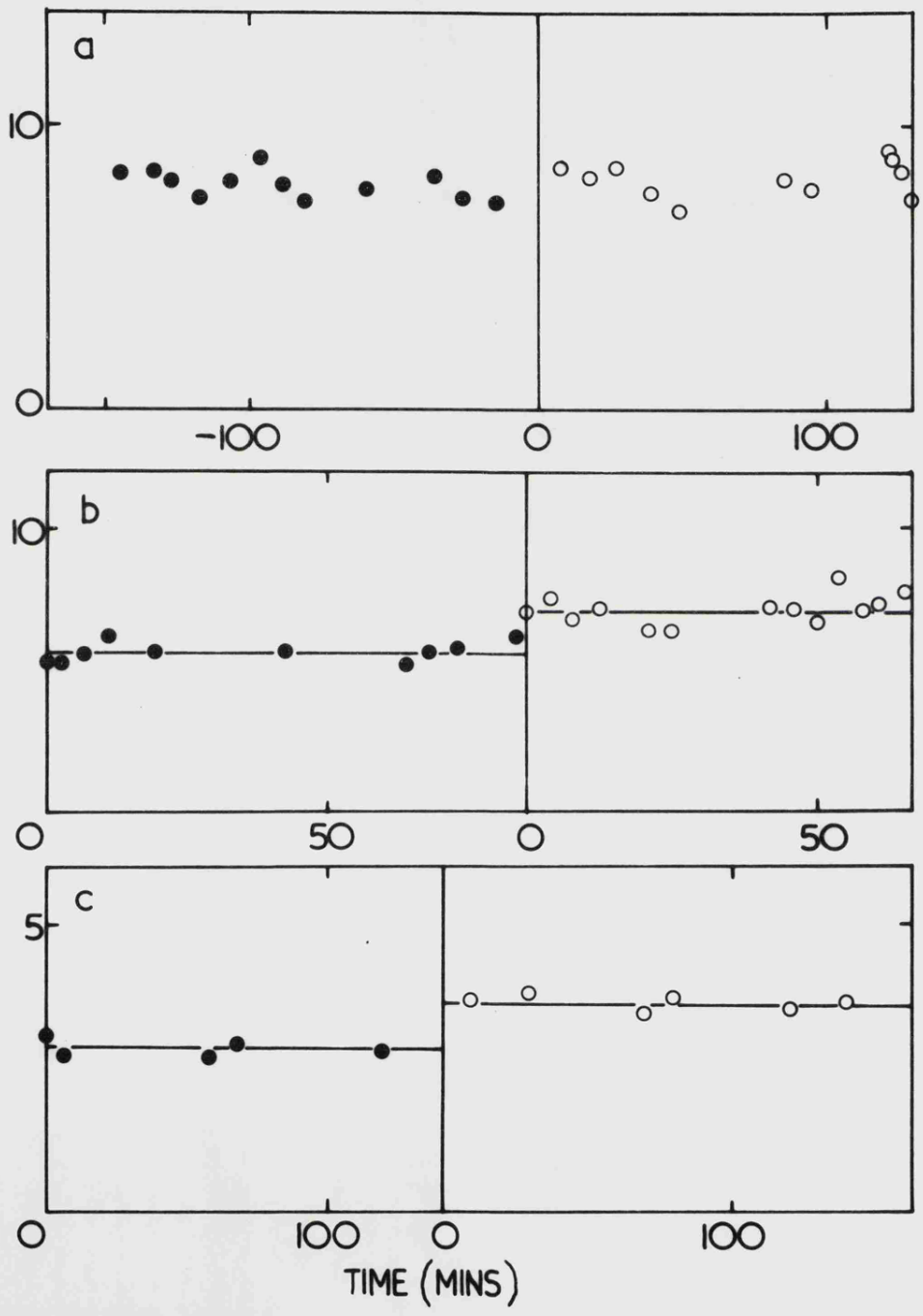

a
10
0
-100
0
100
b
10
0
50
0
50
c
5
0
100
0
100
TIME (MINS)

FIGURE 23.

EFFECT OF THYMINE CONCENTRATION ON THE LEVELS OF TRYPTOPHAN SYNTHETASE.

a The levels of tryptophan synthetase in MC32 (trp R^-) (■,▲) (two separate experiments) and MC17 (trp R^+) (●) growing in 1 μgm/ml.thymine.

b The levels of tryptophan synthetase in MC32 (□,△) and MC17 (○), growing in 40 μgm/ml.thymine.

It should be noted here that the activity of tryptophan synthetase shown has been calculated simply as the number of μmoles of indole used during the reaction period, and the incubation time is not considered. The trpR^- extracts were incubated with substrate for 30 mins whereas the trpR^+ extracts were incubated with substrate for 90 mins

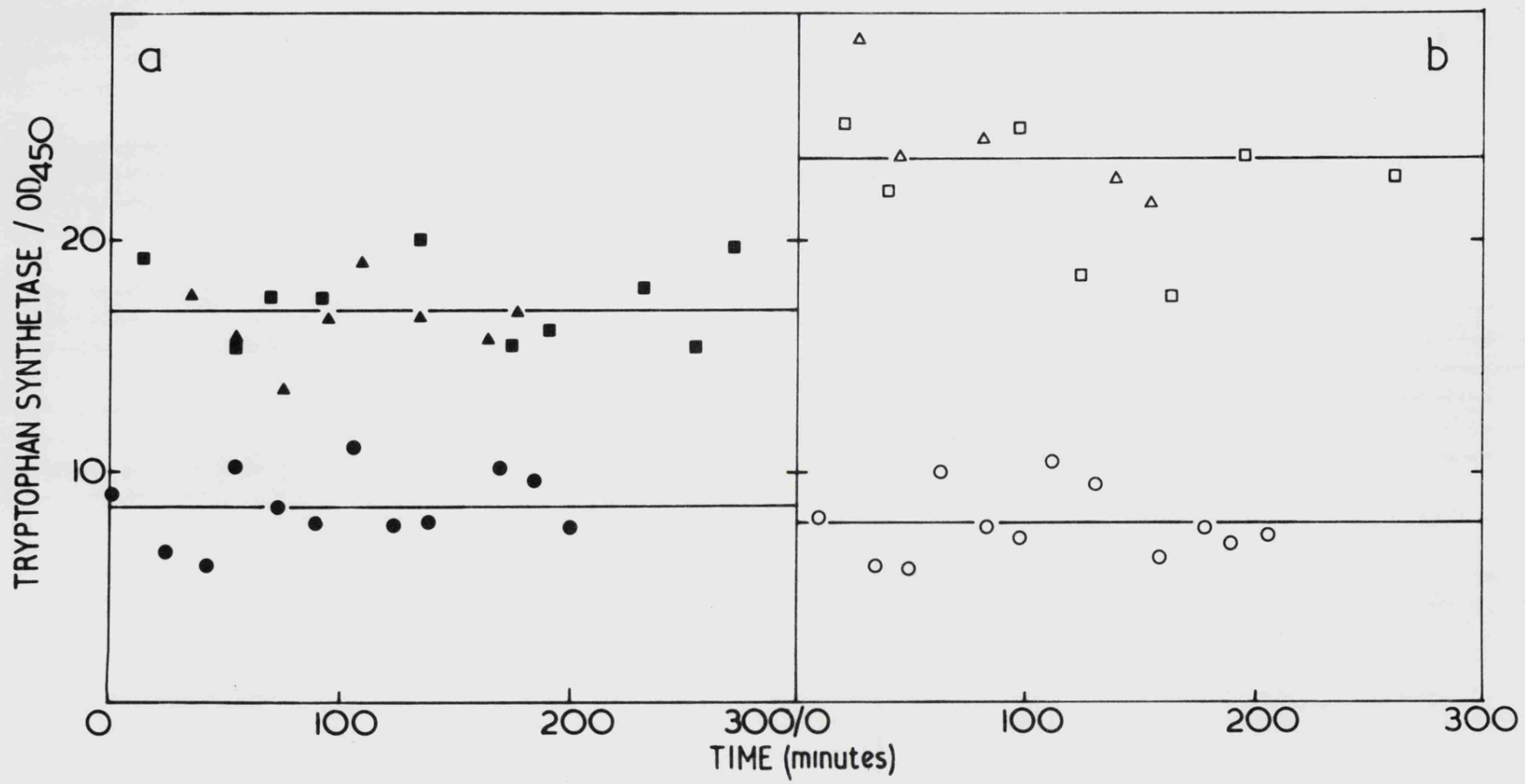
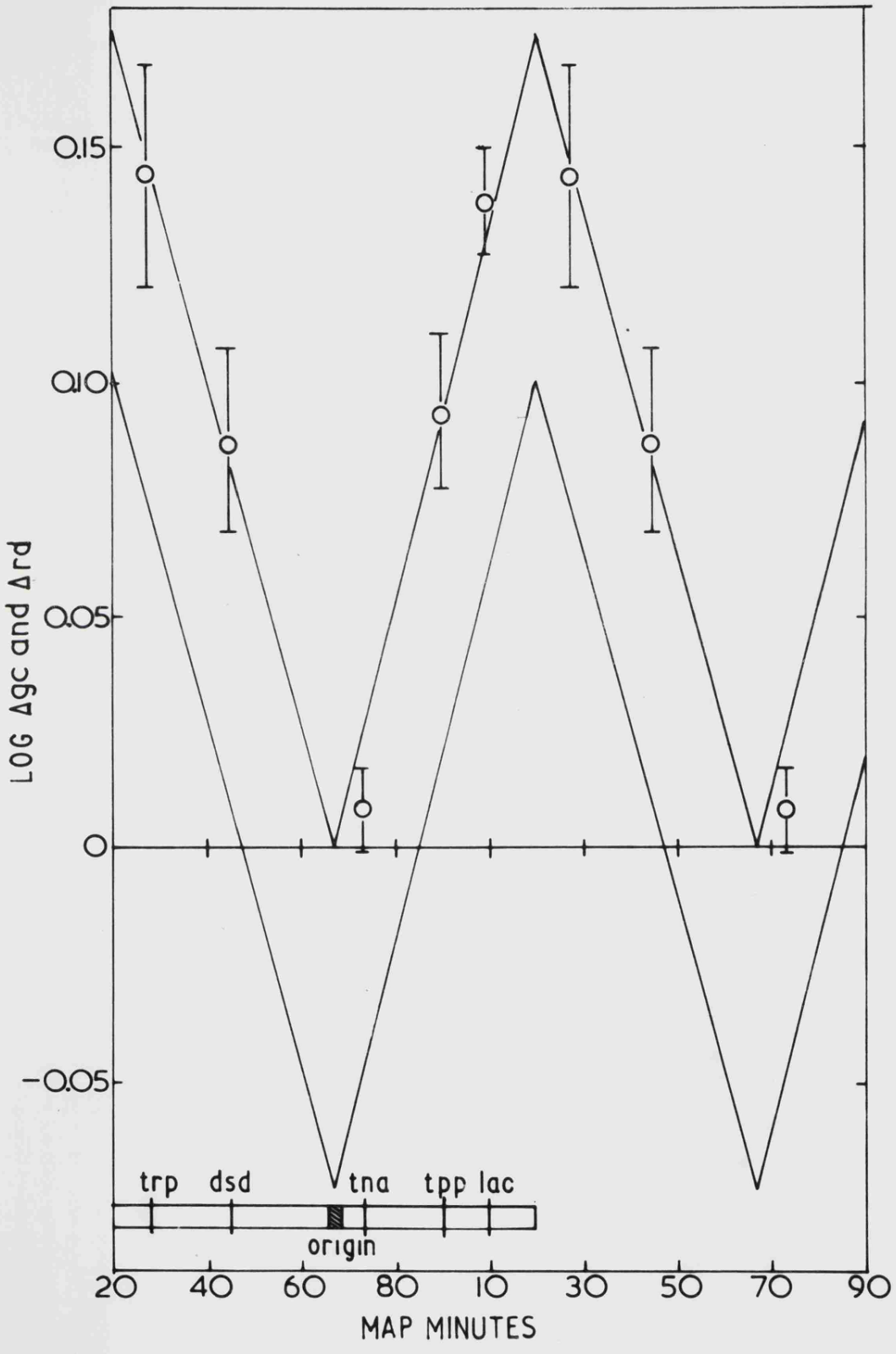

a
b
TRYPTOPHAN SYNTHETASE / OD450
20
10
0
100
200
300/0
100
200
300
TIME (minutes)

FIGURE 24.

CHANGE IN GENE OUTPUT AS A FUNCTION OF GENE LOCATION.

The points corresponding to β-galactosidase and D-serine deaminase are averages obtained from MC5,MC11 and MC17, no significant differences were found either in enzyme levels or mass doubling time in these strains. The point corresponding to tryptophanase was obtained from MC11 (two experiments) and that for thymidine phosphorylase, from MC17 (one experiment). Tryptophan synthetase was measured in MC32 (two experiments). All strains had a mass doubling time of 55 mins in the medium used.

The lower curve is the theoretical curve for the change in relative gene dosage calculated from equation (12). The values used are the same as those for the upper curve with C in high thymine assumed to be 45 minutes. The error bars indicate the standard error of the mean of each measurement.


0.15
0.10
0.05
0
−0.05
LOG Δgc and Δrd
trp
dsd
tna
tpp
lac
origin
20
40
60
80
10
30
50
70
90
MAP MINUTES

chromosome replication from an origin close to the gene specifying tryptophanase. This conclusion is valid on either a gene concentration or relative gene dosage model for gene output since both models predict that a minimum change (either zero or negative respectively) will occur in gene output for genes situated at the origin. The failure to detect a significant negative change in output for any of the genes tested however, suggests that a gene concentration rather than a relative gene dosage model is the more likely.

3. IV. THE CHANGE IN REPLICATION VELOCITY AND THE LOCATION OF THE ORIGIN.

If the observed change in gene output is due to a corresponding change in gene concentration, it is possible to calculate both the location of the origin of replication and the change in replication velocity imposed under the conditions used.

It follows from equation (8) that the ratio of the change in gene concentration of two different genes for a given change in replication velocity is a measure of their relative distances from the origin:-

$$\log(\Delta gc)_a / \log(\Delta gc)_b = x_a / x_b \qquad \text{---------(14)}$$

where $(\Delta gc)_a$ is the change in gene concentration of a gene a whose relative distance from the origin is x_a and $(\Delta gc)_b$ is the change in gene concentration of a gene b whose relative distance from the origin is x_b. This ratio is independent of the absolute values of the replication velocity, the change in velocity and the mass doubling

time as long as these remain constant from experiment to experiment.

It is also possible to obtain an independent expression relating x_a, x_b and the map distance between the two genes. I assume here that replication is bidirectional and symmetrical and define a value d as the shortest distance between two genes. Because the E.coli chromosome is circular and is, by convention divided into 90 map minutes, there are of course constraints on d such that $d \leqslant$ 45 map minutes.

The two genes can be located on opposite arms (that is, one on each replicating unit) or on the same arm of the chromosome. If they are situated on opposite arms, there are two possible values for $x_a + x_b$ (that is, the sum of their distances from the origin):-

$$x_a + x_b = d \qquad \text{---------- (15)}$$

or $$x_a + x_b = 90 - d \qquad \text{---------- (16)}$$

If they are located on the same arm of the chromosome, there are again two values:-

$$x_a - x_b = d \qquad \text{---------- (17)}$$

or $$x_a - x_b = 90 - d \qquad \text{---------- (18)}$$

However, if replication is bidirectional and symmetrical, each arm would be equivalent to 45 map minutes and if gene b is closer to the origin than gene a (setting $x_a > x_b$), then:-

$$0 \leqslant x_a - x_b \leqslant 45 \qquad \text{---------- (19)}$$

Since $d \leqslant 45$ minutes, equation (18) can only yield a meaningful solution if $x_a - x_b = d = 45$. This would indicate that gene b

was located at the origin and gene a at the terminus and is obviously a limiting case. We are thus left with three likely solutions for any pair of genes. The significance of equations (15), (16), (17) and (18) is shown schematically in figure 25.

If we use the observed changes in gene output as a measure of the changes in gene concentration, we can, by pairwise combinations of the genes, obtain separate, although not completely independent estimates for the position of the origin. In order to clarify the treatment, I will take as an example the changes in output of thymidine phosphorylase relative to that of β-galactosidase during a 1 - 40 μgm thymine step. Using the values in table 2:-

$$\log(\Delta gc)_{lac}/\log(\Delta gc)_{tpp} = x_{lac}/x_{tp_p} = 1.48 \qquad \text{-----} \quad (20)$$

If both genes are on opposite arms of the chromosome then from equations (15) and (16):-

$$x_{lac} + x_{tpp} = 9.5 \text{ or } 80.5 \qquad \text{----------} \quad (21)$$

Solving for x_{tpp} yields values of about 3.5 and 32 minutes. This places the origin at 3 or 57.5 minutes.

If both genes are on the same arm and tpp is closer to the origin than lac ($x_{lac}/x_{tpp} > 1$), then from equation (17):-

$$x_{lac} - x_{tpp} = 9.5 \qquad \text{----------} \quad (22)$$

Again, solving for tpp using equations (20) and (22) yields a value of about 19 minutes and places the origin at 70 minutes.

FIGURE 25.

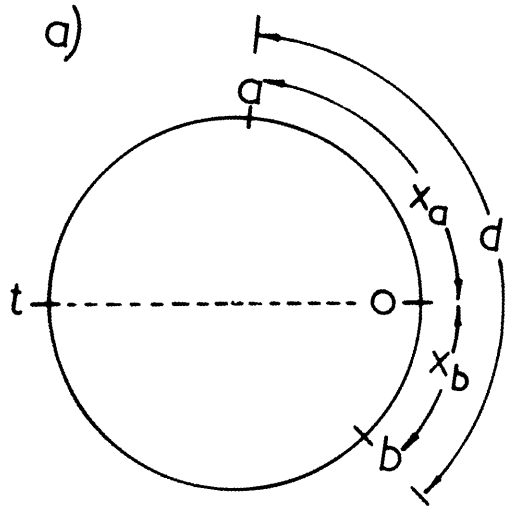
POSSIBLE CONFIGURATION OF TWO GENES WITH RESPECT TO THE ORIGIN.

a The two genes are on opposite arms of the chromosome and the sum of their distances from the origin is less than 45 map minutes.

b The two genes are on opposite arms of the chromosome and the sum of their distances from the origin is greater than 45 map minutes.

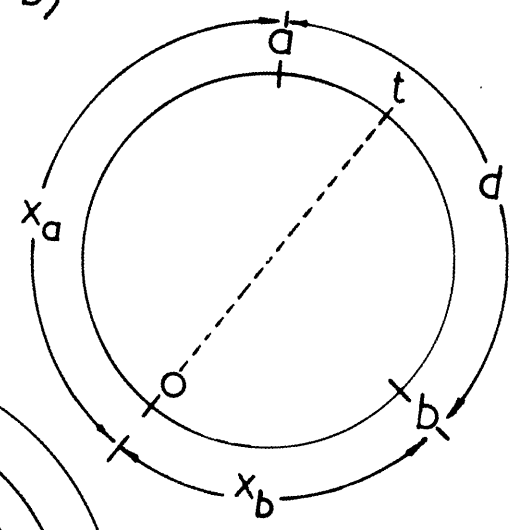
c The two genes are on the same arm of the chromosome.

a)



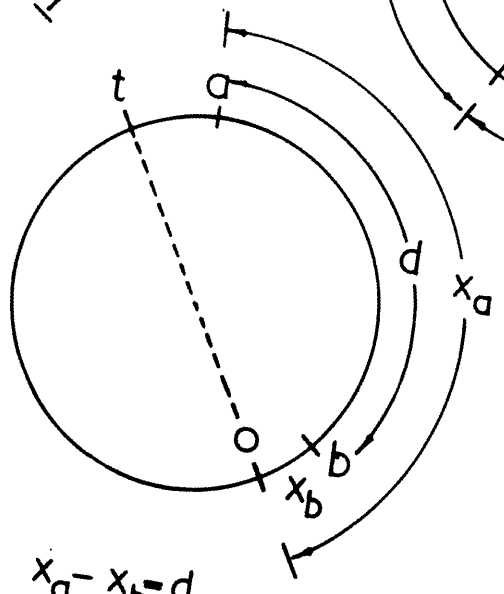
$x_a + x_b = d$

b)



$x_a + x_b = 90 - d$

c)



$x_a - x_b = d$

Comparison of these values with the results from other loci should yield a unique solution for the location of the origin if the assumption that the change in gene output is proportional to the change in gene concentration is correct.

The results of these calculations are presented in table 3. All possible combinations are shown except those involving tryptophanase, since the change in output in this case is too small to measure accurately. It can be seen that one of the solutions for each of the pairwise combinations tested falls between 63.5 and 70.5 minutes on the standard E.coli linkage map. The average value being about 67 minutes. This estimate lies between those obtained for the origin by Masters and Broda (1971) and Bird et.al. (1972) (65 and 74 minutes respectively), both groups using entirely different techniques.

We are now in a position to estimate the change in transit time (ΔC) which occurs over the range of thymine concentrations used here. Using the observed change in gene output, the observed mass doubling time of the cultures and the position of the origin which has been determined above to calculate the value x in equation (8), we can calculate ΔC during the thymine step. The results for each locus during a 1 - 40 μgm thymine step are shown in table 4. The change in gene output for all genes except tryptophanase, corresponds to a ΔC of about 34 minutes. As stated previously, the anomolous results for tryptophanase are not unexpected since, if this locus is near the

TABLE 3 LOCATION OF THE CHROMOSOME ORIGIN DETERMINED FROM THE RELATIVE CHANGE IN OUTPUT OF PAIRS OF GENES WITH KNOWN MAP POSITION

a) 1-40 μgm thymine step

	x_a	trp			lac		tpp
	x_b	lac	tpp	dsd	tpp	dsd	dsd
$x_a + x_b = d$		18	10	38	3.5	30.8	66
$x_a + x_b = 90-d$		63.5	65.0	72	58.5	65.5	23
$x_a - x_b = d$		-	-	70.5	70	-	-

b) 1-20 μgm thymine step

	x_a	lac
	x_b	dsd
$x_a + x_b = d$		32
$x_a + x_b = 90-d$		66
$x_a - x_b = d$		-

The figures are the estimated locations of the origin. The first row assumes that the origin lies between the two genes on the shortest arc. Row 2 assumes that the origin lies between the two genes on the longest arc and rwo 3 assumes that both genes lie on the same arc of the chromosome. The most consistent solutions are underlined.

TABLE 4.

ESTIMATION OF ΔC FROM THE CHANGE IN OUTPUT OF FIVE GENES

	LOCUS				
	lac	trp	dsd	tpp	tna
ΔC	34.5	30	32	34	12

The values given are for ΔC in minutes calculated from a 1-40 μgm.thymine step assuming that the chromosome origin is at map position 67 minutes. (τ = 55 mins)

origin, it will undergo only a small change in concentration and thus be subject to a large percentage error. The calculations presented above are again based on the assumption that gene output is proportional to gene concentration. This assumption would be further corroborated if it could be shown that ΔC calculated by this method is concordant with an independent estimate of ΔC. An independent estimate has been obtained (section 3.1) by measurement of the change in $\bar{G}/\bar{M}$ during the same thymine step and for cells growing at the same rate. The value obtained was about 36 minutes and is in good agreement with those calculated from the change in gene output on a gene concentration model.

Figure 24 (upper curve) shows the theoretical change in gene concentration. It assumes an origin at 67 minutes, a mass doubling time of 55 minutes and a change in transit time of 34 minutes (the value obtained from the enzyme measurements).

3. V. GENE DOSAGE, RELATIVE GENE DOSAGE AND GENE CONCENTRATION

I have shown so far that the data are in close quantitative agreement with the idea that gene output is proportional to gene concentration. I have not, however, indicated to what degree the data are incompatible with a gene dosage or relative gene dosage model.

As has been noted previously (see section 3.1) cell size in these strains, growing with a mass doubling time of 55 minutes in 1 µgm per ml of thymine, increases continuously at a rate approximately

$8 \times OD_{450}/10^9$ cells per 100 minutes (this value was obtained from a least squares fit to the data in figure 17a). If gene output were related to gene dosage, one would expect the enzyme/mass ratios for cells growing in low thymine (for example, the left hand section of figures 19 and 20) to increase at the same rate, since the dosage of any gene, irrespective of position, must also increase at this rate. Comparison of the left hand section of figures 19 and 20 with figure 17a, shows that this is not the case and thus it may be concluded that gene dosage is not a contributory factor in gene output.

In contrast to a gene concentration model, calculations of ΔC on the basis of the assumption that gene output is determined by relative gene dosage, requires that the absolute value of C at one of the thymine concentrations used be known. This can be seen by inspection of equation (12). The transit time of the strain used in these experiments in medium containing high thymine (40 μgm/ml) is unlikely to be outside the range 45 - 65 minutes (Zaritsky and Pritchard 1971) and I have therefore calculated the change in relative gene dosage as a function of the map position for a change in transit time from 45 to 81 minutes and from 65 to 101 minutes. The results of this calculation are shown in the lower two curves of figure 3. The similarity of the two curves shows that the expected change in relative gene dosage is very insensitive to errors in the assumed value of C at high thymine concentrations.

The gene concentration and relative gene dosage curves are quite distinct. If gene output were governed by the relative gene dosage, one would expect there to be a decrease in the output of genes situated on just under half of the replicon. In terms of figure 24, this would mean that we would expect a minimum value of about -0.07 to -0.08 for a gene situated at the origin (lower curve). The experimental values are in very poor agreement with those predicted on the hypothesis that gene output is a function of the relative gene dosage.

3. VI. THE EFFECT OF CHANGES IN CONCENTRATION ON THE OUTPUT OF A GENE SUBJECT TO REGULATION BY AN INTRACELLULAR EFFECTOR

Since the average DNA concentration in a steady-state exponential culture can be significantly reduced by decreasing the replication velocity without any affect on the mass doubling time of the culture, the total rate of protein synthesis must be independent of the overall gene concentration provided of course that the absorbancy accurately reflects the amount of protein per unit volume of culture (see figure 16). Presumably therefore, the decrease in concentration of those genes which contribute significantly to total protein is compensated by a proportional increase in the rate of synthesis per gene copy of their products. In other words, the majority of genes must normally be under some degree of repression by specific internal effectors.

I have tested this notion in the case of the gene specifying tryptophan synthetase. A decrease in the transit time of 34 minutes results in a 40% increase in the differential rate of synthesis of tryptophan synthetase in a *trpR*$^-$ derivative of these strains (figure 23 and 24). If the differential rate of synthesis of this enzyme is measured in the isogenic *trpR*$^+$ parent growing in the same medium (which contains no tryptophan), with a comparable mass doubling time and over the same range of thymine concentrations, no change can be detected (figure 23). This difference in the behaviour of *trpR*$^-$ and *trpR*$^+$ strains shows convincingly that in the *trpR*$^+$ strains, the output of tryptophan synthetase is being modulated by the intracellular concentration of tryptophan and is buffered against changes in gene concentration.

The levels of tryptophan synthetase shown in figure 23 have been calculated simply as the number of μmoles of indole used during the reaction period, and the incubation time is not considered. Since I am concerned only with the relative activities this has no effect on the interpretation of the results. In this regard however, it should be noted that the *trpR*$^-$ extracts were incubated with substrate for 30 minutes whereas the *trpR*$^+$ extracts were incubated for 90 minutes. If this is taken into account, then the enzyme activities in the *trpR*$^+$ strain are between 10 and 20% of the levels in the isogenic *trpR*$^-$ strain. The relative levels are in agreement with those found

by Ito and Crawford (1965) and Rose, Mosteller and Yanofsky (1970) for *trpR*$^-$ and *trpR*$^+$ strains growing in the absence of tryptophan.

3. DISCUSSION

Kacser (1963) has underlined the importance in the elucidation of biological behaviour of "determining the causal connections of a system without isolating the steps of which it is composed". He argues that, although isolation of the component elements yields information concerning the structure of the system, the behaviour of the system cannot be considered simply as the sum of the behaviour of the individual parts since interactions between elements will lead to modifications in behaviour.

This principle has been recognised and applied for many years in studies on the control of bacterial cell growth (e.g. Schaechter et.al. 1958) and, as described in section 1.II and IV, it has yielded useful information concerning the relationship between different classes of macromolecule within the bacterial cell. In this way, it has been shown that for E.coli growing with doubling times of less than 70 minutes, the rate of chromosomal DNA synthesis is determined primarily by the frequency of initiation of new rounds of replication and that this in turn is governed by the rate of mass increase (Donachie 1968; Pritchard et.al. 1969). A more fundamental problem is the way in which the rate of mass increase is maintained in a medium of a particular composition.

One approach to this problem is to analyse the rate of synthesis of various gene products as a function of growth rate. There would seem to be two important factors to consider here. The first, and

possibly the most important, is the change in the activity of general and specific control mechanisms operating both at the level of the gene (e.g. feedback repression) and at the level of enzyme activity (e.g. feedback inhibition). These Maaløe (1969) has called "fine adjustments". The second is the "number" of copies of the individual genes. As described in section 1.IV and VIII, gene number may be equated with three separate and quite distinct parameters: gene dosage, gene concentration and relative gene dosage, all of which change in different ways with growth rate. Quantitation of the "fine adjustments" which "determine and stabilise the growth rate characteristic of a particular growth medium" (Maaløe 1969) are unfortunately complicated by changes in the chromosome configuration which are necessarily associated with changes in the growth rate. The effect of changes in the chromosome configuration on the three quantities describing gene number and the effect of the changes in these quantities on gene output has not previously been investigated. Any analysis of the contribution of "fine adjustments" to the maintenance of growth must take these effects into account. Conversely, quantitation of the effects of changes in gene dosage, gene concentration and relative gene dosage on gene output with growth rate is complicated by changes in the activity of the control mechanisms operating.

In order to separate these two factors, and to determine whether gene output is dependant on gene dosage, relative gene dosage or gene concentration, I have made use of the fact that changes in chromosome

configuration analogous to those brought about by changes in the growth rate, may be obtained by changes in the chromosome replication velocity. This can be achieved without concomitant changes in the growth rate or a significant change in the composition of the growth medium. The results obtained from measurement of the differential rate of synthesis of proteins specified by genes which are maximally repressed or derepressed are in good quantitative agreement with the hypothesis that the differential rate of synthesis is proportional to the average gene concentration in a random population of cells in a steady-state of exponential growth. They are not consistent with the hypotheses that the differential rate of synthesis is affected by relative gene dosage or by gene dosage.

A fundamental assumption underlying this conclusion is that the initiation mass is not affected by the concentration of thymine in the growth medium. Evidence that this is not the case has been presented previously (Zaritsky and Pritchard 1971) and the absence of a significant effect of thymine concentration on the differential rate of synthesis of tryptophanase lends further qualitative support to this assumption, since the gene specifying this enzyme is close to the chromosome origin as determined by Masters and Broda (1971), Bird et.al. (1972) and Hohfeld and Vielmetter (1973).

Since the specific rate of synthesis (that is, the rate per gene) of proteins specified by genes in a fully induced or fully repressed state is not affected by changes in their concentration or relative

dosage, it can be deduced that the concentration of any rate limiting element involved in transcription or translation which is shared by all genes is similarly not affected. This implies that the majority of genes which contribute significantly to net protein synthesis during cell growth must be subject to regulation by specific cytoplasmic effectors such that an increase in their concentration or relative dosage is counterbalanced by an increase in the degree of repression which operates on them. If this were not so, then one would expect the change in relative gene dosage resulting from a change in replication velocity to change the relative proportions of the various gene products. Alternatively, if all common elements involved in transcription and translation were present in saturating concentrations, the same conclusion would be valid since, in this case, an increase in the DNA concentration would lead to an increase in the overall rate of protein synthesis unless the majority of genes were regulated. This point is perhaps more easily seen by recalling (Zaritsky and Pritchard 1971) that in E.coli $15T^-$, a decrease of nearly 50% in total DNA concentration can be imposed by reducing the concentration of thymine in the growth medium without a detectable reduction in the rate of mass increase. Clearly, the majority of gene products cannot normally be undergoing synthesis at the maximal attainable rate per gene and must be under some degree of repression.

In the case of tryptophan synthetase, a 40% increase in gene

concentration in a *trp*R$^+$ strain leads to no detectable change in the differential rate of synthesis of the enzyme (figure 23). The increase in gene concentration must therefore have led to an increase in the intracellular levels of tryptophan and a reduction in the specific rate of transcription and/or translation. In this context it is interesting to recall that certain of the early enzymes in the *trp* biosynthetic pathway are also subject to feedback inhibition by tryptophan (Trudinger and Cohen 1956; Moyed and Friedman 1959). The absence of any detectable difference in the differential rate of synthesis of tryptophan synthetase when the gene concentration is increased implies that feedback inhibition does not play an important role in determining the steady-state rate of synthesis of tryptophan when this amino acid is not present in the growth medium. If it were to do so, then the increase in intracellular concentration of tryptophan which led to an unchanged rate of synthesis of the enzyme would have reduced the flux of tryptophan and hence the growth rate. An obvious explanation for this is that feedback inhibition is important in buffering the cell against short term fluctuations in the environment whereas feedback repression becomes more important in the long term in situations in which the mean constitution of the environment changes. The "relaxation time" of the former mechanism being much shorter than that of the latter.

With regard to the estimate of the location of the origin of

replication (section 3.IV), I should like to emphasise that the calculations do not take into consideration the standard error of the mean of each point shown in figure 24. For the purposes of the arguments concerning gene concentration and gene output, this omission is unimportant. As I have indicated, calculation based on the mean values results in a series of estimates having a spread of some 8 map minutes. The method used in this calculation makes an estimate of the errors involved difficult. An alternative way in which the error might be estimated is by linear regression analysis. However, it would be inappropriate to fit separately, a straight line to points on each "arm" of the map since there are too few data. In order to obtain a more accurate estimate of the position of the origin, it would be necessary to measure the change in output of several additional genes.

To summarise, I have compared the rate of enzyme synthesis from five genes in a fully repressed or induced state and one in an autogenous state (Kuempel et.al. 1965) in cultures growing at the same rate but having different transit times. The data permit the following conclusions:

(1) The initiation mass is independent of the transit time of a replication fork at one growth rate.

(2) The chromosome origin is near the gene coding for tryptophanase and the terminus near the gene specifying tryptophan synthetase. Replication is bidirectional. The data place the origin in the region of 67 minutes on the E.coli linkage map.

(3) The output of genes in a fully induced or fully repressed state is proportional to gene concentration and independent of relative gene dosage.

(4) The output per gene under conditions when it is subject to regulation by a cytoplasmic effector decreases as its concentration is increased.

The data provide a firm foundation for studies in which gene concentration is estimated from gene output and the technique is used to investigate the nature of the control of F replication in the following section.

4. THE REPLICATION OF THE SEX FACTOR F.

4.I. SOME CONSIDERATIONS ON THE CONTROL OF F REPLICATION.

The number of copies of the F episome per cell is known to be small (Frame and Bishop 1971). F is therefore a member of that class of plasmid whose replication is said to be under stringent control (Kontomichalou et.al. 1970). An important characteristic of F replication is that it occurs at a distinct time in the cell cycle, rather than in a random manner, as judged by the discreet doubling in the potential to produce β-galactosidase specified by an Flac particle in a synchronous population of an Flac strain (Donachie and Masters 1966; Zeuthen and Pato 1971). The signal for F replication thus occurs with a definite periodicity during the cell cycle.

A priori, there are several mechanisms by which the phasing of F replication into the cell cycle might occur. I will consider three in detail here:

a) F replication is coupled to some stage of the chromosome replication cycle. For example, to initiation of new rounds of replication or to termination.

b) F replication occurs at a constant cell mass or multiple of that mass, in a manner similar to the initiation of chromosome replication.

or c) F replication occurs when the surface area of the cell attains a critical value (Marvin 1968).

These three modes of phasing F replication into the cell cycle lead to distinctive predictions as to the behaviour of F under different cultural conditions.

F concentration as a function of the chromosome replication velocity.

I have shown previously how, in steady-state batch cultures, growing at a fixed rate, an increase in the chromosome replication velocity will lead to an increase in the average concentration of most genes, the magnitude of the increase being dependent on the location of the gene on the chromosome. Assuming that the time taken to replicate F is much smaller than C, then for purposes of analysis, F may be considered as a chromosomal gene. Consequently, if the replication of F were coupled directly to the chromosome replication cycle, this temporal association would be reflected in the magnitude of the increase in F concentration (figure 4a) and can be quantified (Section I.VIII). If F replication were coupled to termination of a round of chromosome replication, the F particle would behave as a terminal gene, and as such would undergo a maximal change in concentration. If, on the other hand, F replication were coupled to initiation of new rounds of chromosome replication, it would behave as a gene situated at the origin and would undergo no change in concentration. In this event, no distinction could be made between a) and b) above, since both lead to the same prediction at a fixed growth rate. This can be seen more clearly from the equations relating the average gene

TABLE 5.

μ doublings/hr.	(mins)	diameter (microns)	relative surface area.	length/ width	surface area/ mass.	relative cell mass.(OD_{450}/ 10^7 cells).
2.75	22	1.43	19.05	1.94	3.12	6.1
1.85	32	1.22	12.44	1.69	3.76	3.3
1.0	60	0.93	8.61	2.11	4.78	1.8

The data are taken from Schaechter et.al. (1958). Relative surface areas were calculated assuming that the cell is cylindrical with two hemispherical ends. The cell shape is defined by the length/width ratio. This ratio remains fairly constant with growth rate, and also with replication velocity if the cells are in a balanced state of growth (Zaritsky and Pritchard 1973). It does not remain constant however, under conditions of unbalanced but "normal" growth (Zaritsky and Pritchard 1973).

concentration to the transit time of a replication fork and the relative position of the gene on the chromosome (x) (equation 7). For a gene at the origin, x = 0, and the equation reduces to:-

$$(\bar{F}/\bar{M})_o = 1/k \text{ ---------- (23)}$$

Thus the concentration of genes associated with the origin is independent of both C and τ. This is simply a restatement of the assumption that initiation of chromosome replication occurs at a fixed cell mass.

Similarly, if the F particle behaves as an independent replicon in the sense that its replication is not directly coupled to any specific stage of the chromosome replication cycle (in other words, if F replication obeys the same rules as initiation of chromosome replication) then the concentration of F particles is simply:-

$$(F/M)_F = 1/k_F \text{ ---------- (24)}$$

where k_F is a measure of the initiation mass of F. The ratio of F particles to chromosome origins is then:-

$$(\bar{F}/\bar{M})_F/(\bar{F}/\bar{M})_o = k/k_F \text{ ---------- (25)}$$

and will reflect the ratio of their respective initiation masses. For the case where F replication is coupled to or coincident with chromosome initiation, this ratio would be unity. If F replication does not occur at the time in the cell cycle as chromosome initiation, then this ratio would be greater than unity if it occurs before or less than unity if it occurs after chromosome initiation.

In case c) above, k_F will be a function of cell size. In this

regard, it is of interest to note that according to equation (6), mean cell size is a function of the transit time (C) in addition to the mass doubling time (τ), and in accord with this, it has been shown (Zaritsky and Pritchard 1973) that under conditions of balanced growth, an increase in C results in an increase in mean cell size. Under these conditions, a step-up in replication velocity would be predicted to increase the surface area: mass ratio and thus if F replication (were a function of surface area, one would observe an increase in F concentration. It was further noted by Zaritsky and Pritchard (1973) that under certain conditions, the doubling time for cell number was greater than that for mass. Consequently the cells never reach a balanced state of growth but increase in size continuously (see figure 17). The concentration of F particles would consequently be predicted to fall continuously. Unfortunately, unlike the change in cell size which occurs with growth rate, that brought about by changes in replication velocity also results in changes in cell shape, and thus it is difficult to make quantitative estimates of the change in surface area: mass ratio.

F concentration as a function of the mass doubling time of the culture.

Changes in chromosome configuration analogous to those brought about by increasing the replication velocity can be brought about by decreasing the growth rate of the culture (section 1.VIII and figure 4b). Thus the predictions concerning the behaviour of F with

increasing or decreasing transit time on the three models above should also be true for decreasing or increasing growth rate. In addition, since the changes in cell size associated with changes in growth rate do not seem to be accompanied by significant changes in cell shape (see Table 5), quantitative estimates of the change in surface area: mass ratio become possible.

Although no careful measurements of the variation in cell size with growth rate have been published for E.coli, data have been published for a closely related organism, Salmonella typhimurium (Schaechter et.al. 1958) from which it is possible to calculate relative surface area. Assuming that the cell size changes are similar in both organisms, that the cell is a simple cylinder with two hemispherical ends and that cell mass as measured by the optical density of the culture at 450 nm is proportional to the cell volume, I have calculated, using the data from Schaechter et.al. (1958), the relative surface area and the surface area:mass ratios for cells growing over the range of mass doubling times from 22 to 60 minutes. The results of these calculations are presented in table 5. It can be seen that a decrease in the mass doubling time from 60 to 22 minutes results in a decrease in the relative surface area:mass ratio of approximately 35%. One would thus expect the average concentration of F and consequently the ratio of F concentration: concentration of chromosome origins to decrease by this degree over this range of doubling times if F replication were coupled to a

constant surface area.

F concentration during a shift up in growth rate.

The kinetics of the change in macromolecular composition following a shift-up in growth rate (Kjeldgaard et.al. 1958) has been well characterised and have been analysed formally (Bleecken 1969). Thus the specific rate of mass increase assumes its new value almost immediately following the shift, the specific rate of DNA synthesis after a period equal to the transit time C, and the specific rate of increase in cell number after a period equal to C + D minutes.

The concentration of genes associated with the chromosome origin of course remains constant and if F replication were coupled to or coincident with initiation of chromosome replication or if it occurred at a constant cell mass, the ratio $(\bar{F}/\bar{M})_F/(\bar{F}/\bar{M})_o$ should remain constant throughout the shift. If however, F replication were directly coupled to a later stage of the replication cycle, this ratio would be predicted to fall and reach its final steady-state value Cx minutes after the shift. Similarly, if F replication were coupled to a constant surface area, its concentration would fall, presumably reaching a final steady-state value after C + D minutes (the time at which the new surface area:mass ratio is presumably attained).

The experiments presented in section 4.II were designed to test whether F replication is directly coupled to any stage of the

chromosome replication cycle making use of the theoretical predictions outlined above.

4.II. THE EFFECT OF CHANGES IN REPLICATION VELOCITY ON THE OUTPUT OF β-GALACTOSIDASE SPECIFIED BY AN Flac PARTICLE

The data presented in the previous chapter show that a change in gene concentration results in a proportional change in the differential rate of synthesis of certain enzymes if their structural genes are subject to a constant degree of repression or derepression. At a given growth rate, any change in F concentration would thus be predicted to result in a proportional change in enzyme output from an episomally specified gene which is similarly subject to a constant degree of repression or derepression. Since for a given change in replication velocity, the change in concentration of any gene is dependent on its temporal position in the replication cycle, comparison of the change in output from a chromosomal gene in a fully repressed or derepressed state with that from the same gene when located on an F particle during a transition from one replication velocity to another, should allow a determination to be made of the position in the replication cycle (if any) at which F replicates. If F replicates after the chromosomal gene, then the change in output of the episomal gene will be greater than that of the chromosomal gene and vice versa.

The lac operon is located some two thirds of the length of the replicon from the origin, as judged from the data in figure 24

and the estimates of others (Masters and Broda 1971; Bird et.al. 1972). Its average output changes quite considerably for a change in transit time of about 34 minutes. I have compared the change in output of β-galactosidase specified by a lac gene on an F'lac particle with the changes observed when the gene is situated on the chromosome during a step-up from low to high thymine. For this purpose I have employed two strains: a male harbouring an autonomous F'lac and carrying a deletion of the chromosomal lac region (MC10), and an isogenic female which has a fully functional chromosomal lac region (MC5). The procedures used here are the same as those used in the experiments described in the previous chapter. The results from an experiment with MC10 growing in glycerol cas medium during both a 1-10 and 1-20 μgm thymine step are shown in figure 26a and similar results are shown in figure 26b for a 1-40 μgm thymine step in cells growing in the glycerol amino acid medium. Representative results from the female strain have been presented previously (figure 19). The most striking difference between the two sets of results is that there is no detectable increase in the levels of β-galactosidase during the step when the lac operon is situated on the episome, whereas, the change in output of β-galactosidase in the female strain is quite considerable (about 40% when growing in glycerol cas medium and undergoing a thymine step of 1-20 μgm and about 38% when grown in glycerol amino acid medium during a 1-40 μgm

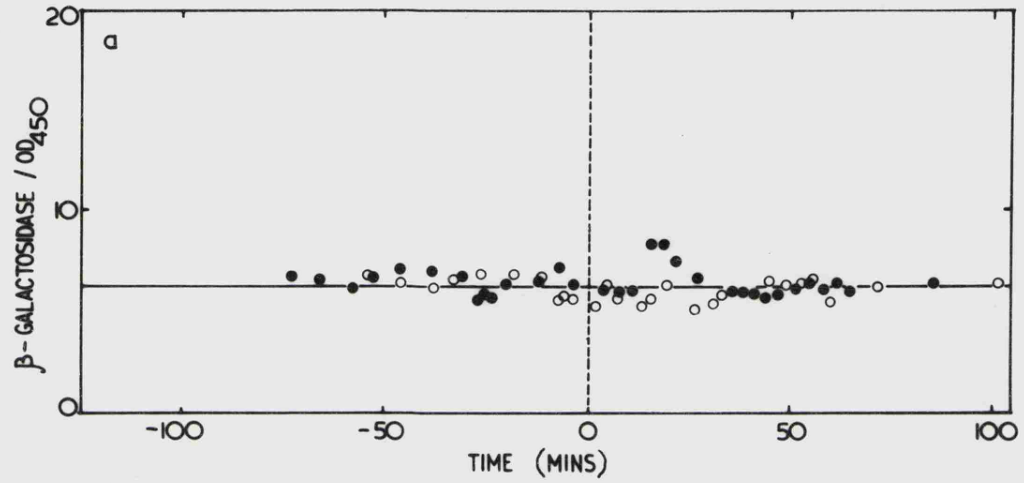
FIGURE 26.

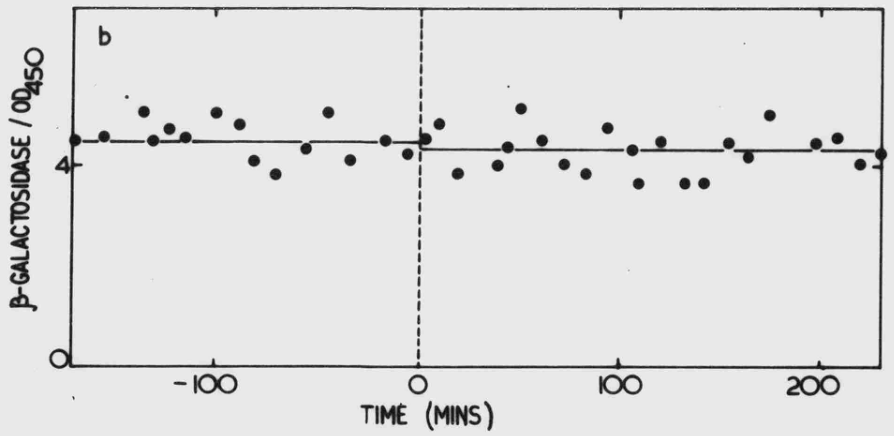
EFFECT OF THYMINE CONCENTRATION ON THE POTENTIAL FOR SYNTHESIS OF β-GALACTOSIDASE SPECIFIED BY AN Flac PARTICLE.

The experimental conditions were the same as those in the experiments shown in figure 19.

a MC10, growing in glycerol Casamino acid medium (τ = 30 mins) and undergoing a step-up from 1-10 µgm/ml (o) and from 1-20 µgm/ml.thymine (•).

b MC10, growing in glycerol amino acid medium (τ = 55 mins) and undergoing a step-up from 1-40 µgm/ml.thymine.


a
β-GALACTOSIDASE / OD_{450}
20
10
0
-100
-50
0
50
100
TIME (MINS)


b
β-GALACTOSIDASE / OD_{450}
4
0
-100
0
100
200
TIME (MINS)

thymine step). It is of interest to note here that the absence of any effect of thymine concentration on β-galactosidase output in the male reinforces the earlier conclusion that the increase in gene output in the female strain is a result of an increase in the concentration of the lac operon rather than any direct effect of thymine concentration on its expression.

The results presented in figure 26 therefore preclude the possibility that F replication is coupled to any part of the chromosome replication cycle other than to initiation. They do not however, distinguish between coupling to initiation or to a constant cell mass.

Since there is a decrease in cell size following a step-up from low to high thymine, and therefore an increase in the surface area: mass ratio, it might seem unlikely that F replication is coupled to a constant surface area. However, since the cell size increases continuously in low thymine (figure 17), and this is accompanied by a change in cell shape (Zaritsky and Pritchard 1973), it is difficult to ascertain the size of the change in F concentration predicted on a model in which F replication is coupled to a constant surface area. It is possible therefore, that a change in F concentration does indeed

occur but that it is obscured by the resolution of this technique.

In order to distinguish between coupling to initiation, to a constant cell mass or to a constant surface area, it would be necessary to obtain a direct measure of the ratio $(\bar{F}/\bar{M})_F/(\bar{F}/\bar{M})_{origin}$ as a function of the mass doubling time of the culture. Since cell size and shape vary in a predictable way with growth rate (Schaechter et.al. 1958), it is then possible to estimate the change in surface area: mass ratio.

Although I have not as yet obtained estimates of $(\bar{F}/\bar{M})_F/(\bar{F}/\bar{M})_{origin}$, an indirect method is evident from the studies on gene concentration and gene output. It assumes that the output of a gene is independent of its position with respect to the episome or chromosome or, more specifically, that any change in the degree of repression or derepression brought about by a change in the growth rate affects the gene equally whether it be located on the chromosome or on the episome. It would then be possible to compare, for example, the levels of β-galactosidase in an F'lac/Δlac

strain with those in an isogenic lac$^+$ female strain growing at the same rate and under the same conditions. This should give a measure of the concentration of the episome relative to that of the chromosomal lac gene. From equation (7), it can be seen that gene concentration is dependant on the three variables C, x and Υ. If both male and female strains are thy$^+$, C may be estimated to be a constant of about 45 minutes (Cooper and Helmstetter 1968), x may be estimated from a knowledge of the position of the origin and Υ will be known. It is thus possible to estimate the concentration of the chromosomal lac operon in terms of k. If the output of a gene is proportional to its concentration then we can write:-

$$dE/dM \propto \bar{F}/\bar{M} \quad \text{or} \quad dE/dM = \frac{k'2}{k}^{-Cx/\Upsilon} \qquad \text{---------- (26)}$$

where dE/dM is the differential rate of enzyme synthesis and k', the constant of proportionality. In physical terms k' is a function of the degree of repression or derepression acting on the gene. Knowing dE/dM, C, x and Υ it is thus possible to obtain a value for k'/k and thus a measure of the expected level of β-galactosidase for x = 0 (i.e. if the lac operon were situated at the chromosome origin). Similarly, the concentration of F particles in terms of the level of β-galactosidase will be:-

$$(dE/dM)_F = k''(\bar{F}/\bar{M})_F \qquad \text{---------- (27)}$$

where k'' is a measure of the degree of repression or derepression acting on the episomal lac operon. If k' = k'', then the level of β-galactosidase specified by the episome compared to that calculated to be specified by the chromosomal lac operon if it were located at the origin will give a measure of the relative concentration of F with respect to the chromosome origin, and, as shown in section 4.I, this relationship as a function of growth rate will behave in a predictable way on both a surface area and a constant mass model. However, published evidence concerning the relative values of k' and k'' is inconclusive and it cannot be assumed therefore, that they are equal over a range of growth rates. Further, in order to equate the relative levels of β-galactosidase with the relative concentrations of the respective genes, as a function of growth rate, even taking into account that k' and k'' may have different values, it would be necessary to assume that the value k''/k' remains constant with growth rate, that is, if k' were to change with growth rate, there must be a proportional change in k''.

4. III. THE EFFECT OF F ON THE INITIATION MASS OF THE CHROMOSOME AND ON THE OUTPUT OF A CHROMOSOMAL GENE.

The existence of an interaction between the mechanism which controls F replication and that which controls the replication of the bacterial chromosome has been well documented. It is known for

example, that when an F particle is integrated within the bacterial chromosome, its replication in general proceeds passively under the control of chromosome replication and that F particles whose replication is prevented at high temperatures can be rescued by integration into the host chromosome (Cuzin and Jacob 1964). It has recently been shown, in addition, that an integrated F particle is able to suppress a temperature sensitive initiation lesion (dnaA) in the host cell, enabling replication of the bacterial chromosome to proceed at the non-permissive temperature. This phenomenon has been called integrative suppression (Nishimura et.al. 1971). Presumably in these strains, at the non-permissive temperature, chromosome replication is to some degree under the control of the F replication system. The mechanism operating here remains obscure at present.

The control of replication of one replicon by the other would seem to require that F be integrated within the bacterial chromosome. However, it seemed possible that the introduction of an F particle into a cell might in some way affect chromosome replication. Indeed, if for example, the introduction of F affects the initiation mass of the chromosome, this would seriously affect the interpretation of data from experiments in which the gene concentration of F relative to that of the chromosomal genes was compared in F' and F^- strains.

The introduction of F might also affect the expression of

chromosomal genes, an effect which would be important in experiments in which gene concentration is estimated by measuring the output of a specific gene in haploid and merodiploid strains.

It will be noted that the DNA concentration of a culture is dependant on both C and τ (equation 1). It is also a function of the chromosome initiation mass (k). Thus if the introduction of F has an effect on the initiation mass of the chromosome, this should be reflected in a difference in the DNA concentration between cultures of the male and female strain if they are both growing at the same rate with identical values of C. I have compared the DNA:mass ratios of two isogenic strains (MC17 and MC84) one of which carried the F'trp episome KLF23 (MC84), growing at the same rate in high thymine. The results are presented in figure 27. Although the degree of scatter is quite high, the mean values for the DNA:mass ratios are in close agreement (28.7 for the F^- and 29.7 for the F'trp strain). I therefore conclude that F has no measurable effect on the chromosome initiation mass. This conclusion relies on the assumption that C in both strains is the same since neither C nor k in equation (1) are known. The contribution of F DNA in the male strain to total DNA would be quite small (based on the approximate map distance covered by the episome, it would be of the order of 5%).

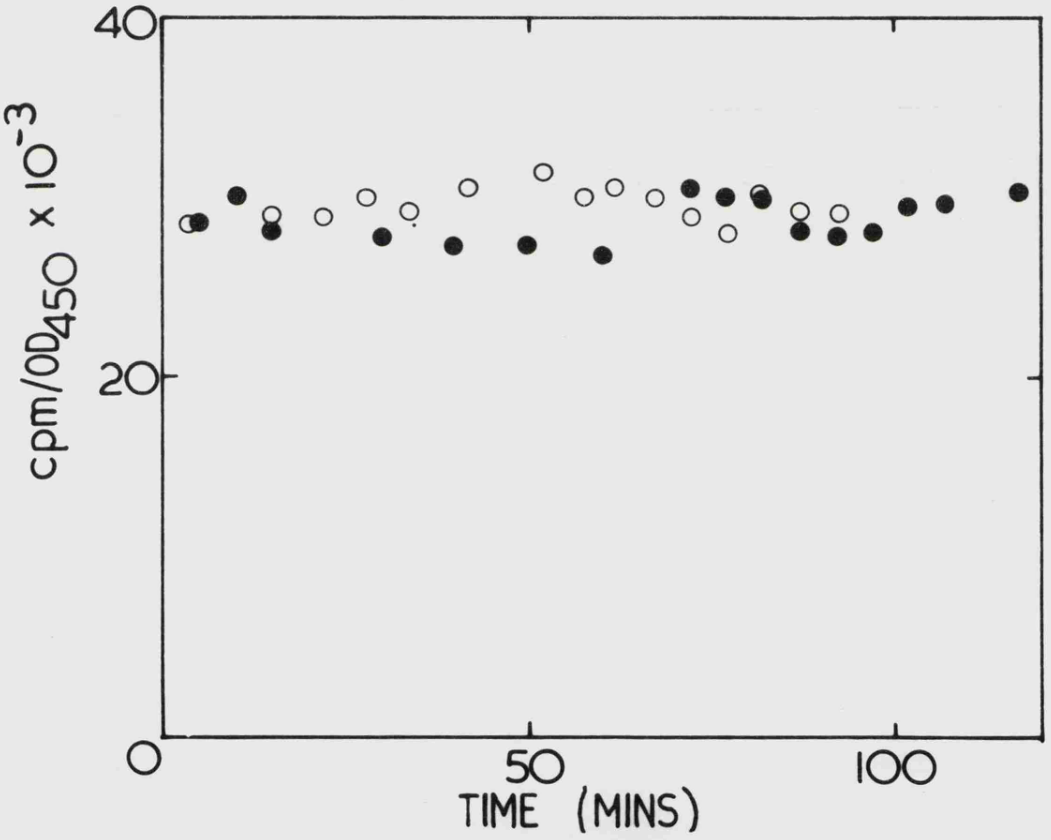
The effect of F on the output of β-galactosidase specified

FIGURE 27.

EFFECT OF THE PRESENCE OF AN F PRIME FACTOR ON THE CHROMOSOME INITIATION MASS.

MC17 (•) and its F'trp derivative, MC84 (o), were grown in glycerol amino acid medium in high thymine (40 μgm/ml). The experimental procedure was the same as for the experiment shown in figure 15. The data is expressed as cpm/ml/OD_{450} x 10^{-3}. Both strains had identical mass doubling times.

The DNA:Mass ratio from the F*trp* derivative might be expected to be slightly higher than that from MC17 due to the presence of the F particle.


40
20
0
50
100
TIME (MINS)
cpm/OD450 x 10-3

by a chromosomal gene has also been studied. I have compared both the basal and induced levels of this enzyme in MC17 and in MC84. The results are presented in figures 28 and 29. Again, as in the case of the DNA:mass ratios, there is no significant difference between the basal activities (2.42 in the F^-, 2.43 in the F'trp strain) or between the induced levels (5.59 in the F^-, 5.34 in the F'trp strain) and it can therefore be concluded that the introduction of F has no effect on the output of β-galactosidase specified by the chromosomal gene. In regard to figures 28 and 29, the values shown are not expressed strictly in terms of enzyme units but simply as OD_{420}/OD_{450}, since I was interested only in relative values.

As was the case with the comparison of the DNA:mass ratios, this conclusion relies on the assumption that C and Υ are the same in both strains under these conditions. Since the differential rate of enzyme synthesis will be affected by changes in C,k', k'' and k (equation 26) and all three are unknown quantities, it is possible that the introduction of F results in changes in any or all these parameters. The changes might be such that their effects on enzyme levels negate each other and result in equal levels in both strains.

I feel that the close agreement between the enzyme level measurements and the measurements of the DNA:mass ratios in these strains argues against any such effect of F on the output of

FIGURE 28.

EFFECT OF AN F*trp* ON THE OUTPUT OF CHROMOSOMAL β-GALACTOSIDASE:BASAL LEVELS.

MC17 (•) and MC84 (o) were grown in glycerol amino acid medium in high thymine (40 μgm/ml.). Data is expressed as enzyme activity (arbitrary units)/ml/OD_{450}. The substrate reaction was allowed to continue overnight at 28°C.

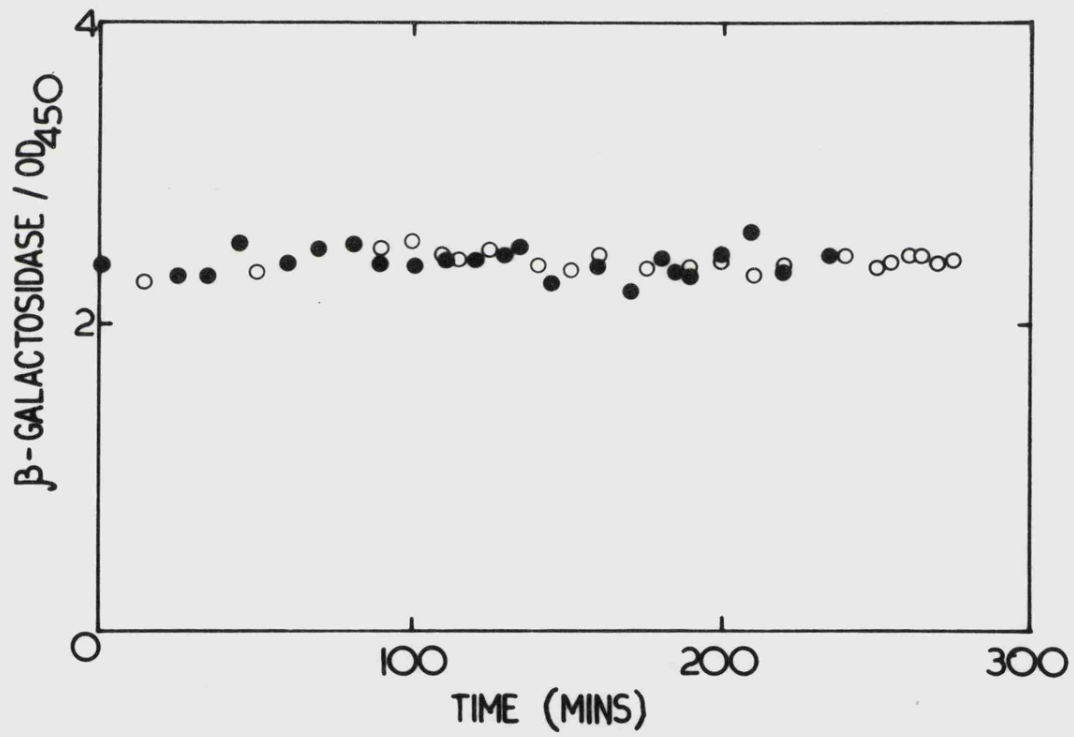
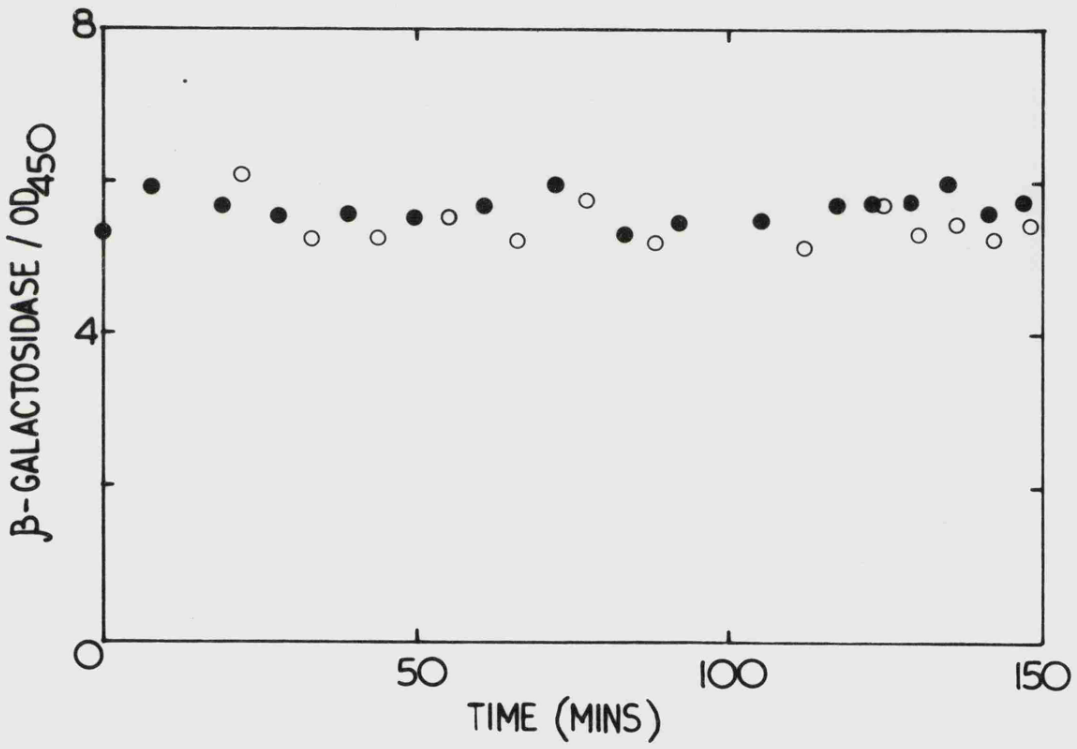

β-GALACTOSIDASE / OD450
4
2
0
100
200
300
TIME (MINS)

FIGURE 29.

THE EFFECT OF AN F*trp* PARTICLE ON THE OUTPUT OF CHROMOSOMAL β-GALACTOSIDASE: POTENTIAL.

The growth conditions used were identical to those used in the experiments shown in figures 27 and 28. MC17 (•); MC84 (o). The induction procedure is described in Materials and Methods.


8
4
0
β-GALACTOSIDASE / OD450
50
100
150
TIME (MINS)

β-galactosidase, on the chromosome replication velocity or on the chromosome initiation mass.

4. IV. THE LEVELS OF TRYPTOPHAN SYNTHETASE IN A $trpR^-$ AND $trpR^+$ MERODIPLOID

It has been shown that the level of tryptophan synthetase in a wild type ($trpR^+$) strain, diploid for the *trp* operon was increased when compared to the haploid parental strain (Stetson and Somerville 1971). This observed difference in tryptophan synthetase levels is difficult to reconcile with my finding (see section 3.VI and figure 23) that a decrease in the transit time of a replication fork of about 34 minutes does not result in a change in the levels of the enzyme in a $trpR^+$ strain, for in both cases there is an increase in the concentration of the *trp* operon. Indeed, if the mass doubling times of the haploid and merodiploid strains are the same, the levels of tryptophan synthetase might also be expected to be equal since it could be argued that the increased concentration of the *trp* operon in the merodiploid would lead to an increased degree of repression via the trpR gene product. This would consequently reduce the rate of tryptophan synthetase production per *trp* gene.

The magnitude of the increase in tryptophan synthetase upon the introduction of an Ftrp particle, it was argued (Stetson and Somerville 1971), indicated that the episomally located *trp* genes tend to specify more enzyme than do chromosomal genes. The

validity of this conclusion, as I will discuss later, depends on the number of episomal trp genes compared to chromosomal genes. In an attempt to resolve the discrepancy between the two sets of results, I have measured the levels of tryptophan synthetase in both a trpR$^+$/Ftrp merodiploid strain (MC84) and an isogenic trpR$^-$/Ftrp derivative (MC85) and have compared these levels to the enzyme levels in their F$^-$ parents under the same experimental conditions as were used in the experiments shown in figure 23. The results of these experiments are presented in figure 30.

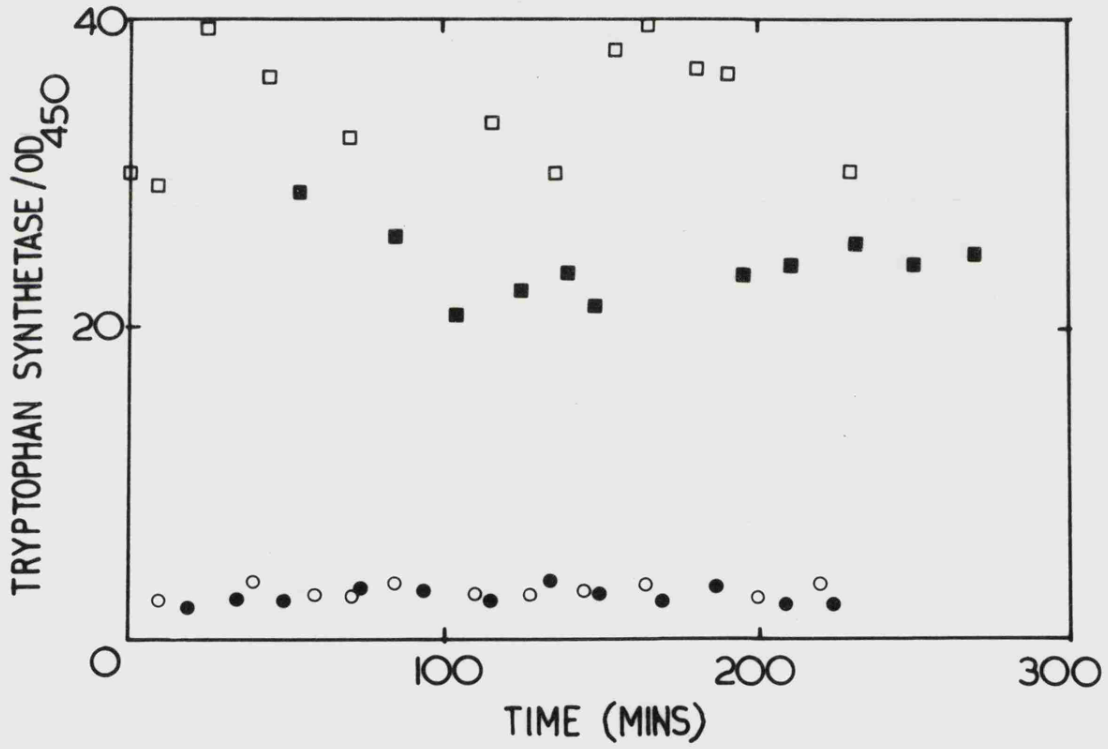
It can be seen that although the enzyme level in MC85 is higher than in its F$^-$ parent (upper two curves), the levels in both the wild type parental strain and its Ftrp derivative are the same. Thus under the conditions used in this study, an increase in the concentration of the trp operon obtained either by an increase in the replication velocity (figure 23) or by the introduction of an Ftrp particle, causes an increase in the level of tryptophan synthetase in the trpR$^-$ strains but has no effect on the levels of this enzyme in the wild type (trpR$^+$) strains.

There are several explanations which might account for the difference between the results presented here and those of Stetson and Somerville. It might be due, for instance to a difference in the behaviour of the episomes used, or to a difference in growth conditions. The Ftrp episome which was used by these authors

FIGURE 30.

TRYPTOPHAN SYNTHETASE IN ISOGENIC *trpR*$^+$/F*trp* and F$^-$ AND *trpR*$^-$/F*trp* F$^-$ STRAINS.

MC17 (●), its F*trp* derivative MC84 (o), MC32 (*trpR*$^-$) (■) and its F*trp* derivative MC85 (□) were grown in glycerol amino acid medium in high thymine. The enzyme assay conditions were the same as used in the experiments reported in figure 23. The data here, unlike those in figure 23 do take into account the difference in the incubation time used for the *trpR* and *trpR*$^-$ strains.


TRYPTOPHAN SYNTHETASE/OD450
40
20
0
100
200
300
TIME (MINS)

was the F'colVcolB trp of Fredericq (Fredericq 1969) and that used here was KLF23 (see Low 1973). It has been shown that FcolVcolB trp can co-exist in the same cell as the classical F factor without demonstrable incompatability (Jackson and Yanofsky quoted in Stetson and Somerville 1971). The behaviour of this episome is therefore somewhat different from that of the classic F and F prime family of plasmids, and the number of copies per cell might be significantly different. Secondly, the growth conditions used here were not strictly comparable to those used by Stetson and Somerville. These authors used stationary phase cultures (although they state that similar results were obtained with exponentially growing cultures) whereas measurements were made in this study on cells growing exponentially in a steady-state and at a comparatively low concentration ($0.1 \leqslant OD_{450} \leqslant 0.2$).

Although in my experiments, the mass doubling time of the trpR$^+$/Ftrp and F$^-$ strains were identical, it was noted that there was a distinct difference in the mass doubling times of the trpR$^-$/Ftrp and F$^-$ strains. It is therefore not possible to relate the difference in enzyme levels in the trpR$^-$ strains to the relative contributions of the enzyme specified by the chromosomal or episomal genes with any confidence.

Further investigation revealed that the mass doubling time of the trpR$^-$/Ftrp strain was dependant on the thymine concentration in

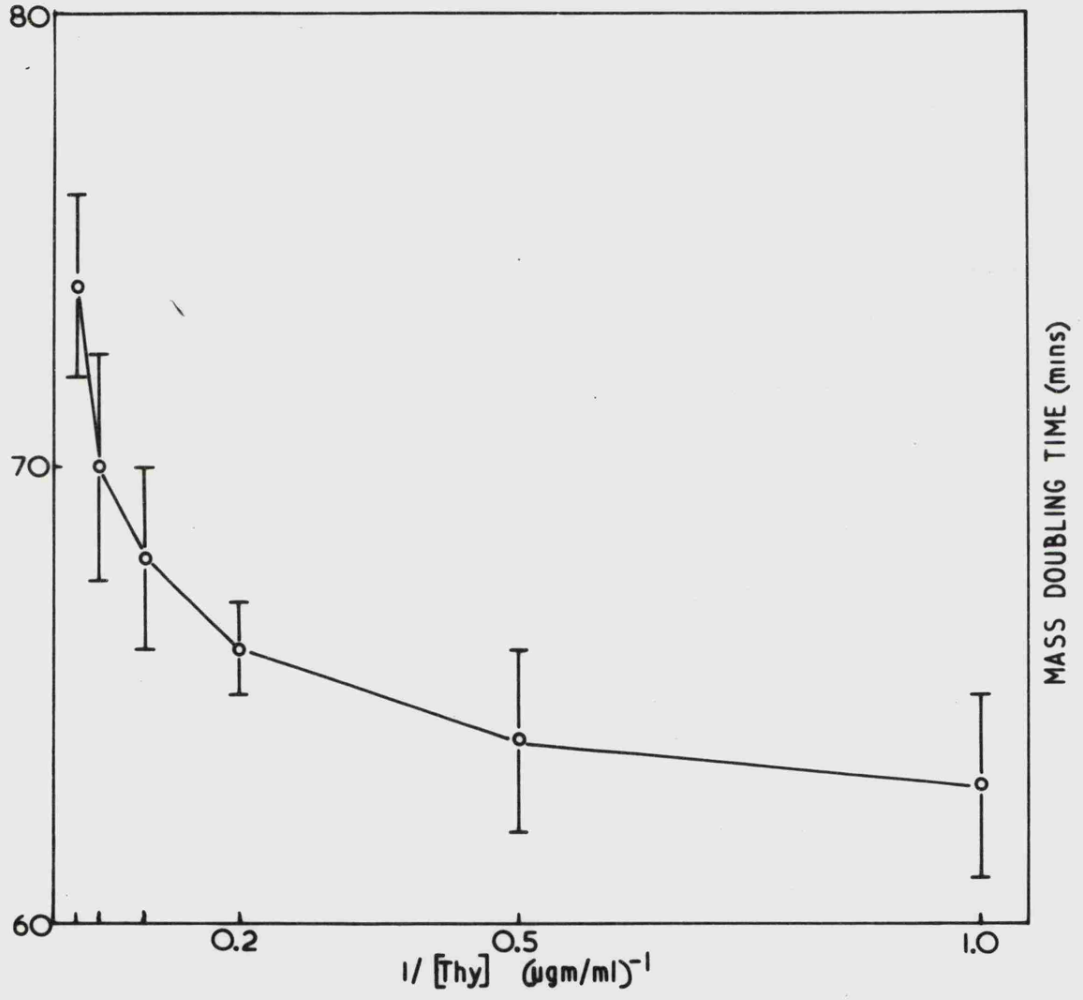
the growth medium. For cells growing in a given medium at 37°C, the mass doubling time was found to increase with increasing thymine concentration. The results are presented in figure 31. Over the range of thymine concentrations from 1 to 40 ugm per ml, the mass doubling time of the culture increased from 63 to 74 minutes. The mass doubling time of this strain growing in 1 µgm of thymine per ml was significantly longer than that of the isogenic F^- parent growing under the same conditions in high or low thymine (τ = 55 minutes). I have not carried this investigation further.

It is perhaps of interest to speculate as to the mechanism underlying this behaviour. The level of tryptophan synthetase in a trpR$^-$ mutant is approximately five to ten fold higher than in its trpR$^+$ counterpart growing in medium without tryptophan (see figure 23). This increase has little effect on the growth rate of the cells (see for example Rose et. al. 1970). The introduction of an Ftrp particle into the trpR$^-$ background further increases the level of tryptophan synthetase and also increases the mass doubling time of the cells. This increase in mass doubling time must result from the increase in the level of tryptophan synthetase or more specifically, the enzymes of the trp operon since both enzyme levels and mass doubling times of the Ftrp and F^- wild type strains are identical under these conditions.

FIGURE 31.

EFFECT OF THYMINE CONCENTRATION ON THE GROWTH RATE OF MC85.

The cells were grown in glycerol amino acid medium containing various concentrations of thymine. Growth was followed for at least three generations.


80
70
60
0.2
0.5
1.0
1/ [Thy] (µgm/ml)⁻¹
MASS DOUBLING TIME (mins)

There would seem to be two possible explanations for this:

a) that the increase in tryptophan synthetase production significantly reduces the fraction of total energy available to the cell for other biosynthetic reactions.

or b) that the increase in enzyme levels leads to an increase in the intracellular concentration of an intermediate in the biosnthesis of tryptophan which is inhibitory for growth.

On either hypothesis, the greater the differential rate of enzyme synthesis, the greater would be the predicted increase in mass doubling time. This is indeed what is observed. An increase in replication velocity resulting from an increase in thymine concentration increases the concentration (and therefore the differential rate of synthesis of tryptophan enzymes) of the chromosomal trp operon (figure 23) whilst not affecting the concentration of the episomal genes (figure 26). The corresponding increase in mass doubling time tends to reduce the concentration of the chromosomal trp operon. This effect is greater the higher the replication velocity and, from figure 31, seems to approach a minimum asymtotically.

4. DISCUSSION.

Present knowledge concerning the molecular mechanisms which govern replication of the bacterial chromosome and that of stable autonomously replicating episomes is limited. Several models have been proposed to explain various aspects of the processes leading to initiation of rounds of replication. Three such models have been briefly outlined previously (section 1.II and VI). They can be divided into two basic types. The first class of model proposes that initiation is controlled in a positive manner. It includes membrane site models such as the replicon hypothesis of Jacob et.al. (1963); "initiator" accumulation models such as that of Helmstetter et.al. (1968), and a modification of the latter which proposes a requirement for the maturation of an initiation complex for initiation to take place (Bleecken 1969). The second class proposes that initiation is controlled in a negative fashion. The inhibitor dilution model of Pritchard et.al. (1969) and the "site territory" model (Marvin 1968) fall into this category.

A premise first formulated in the replicon hypothesis and subsequently encompassed in more formal models of both a positive and negative nature, is that initiation of chromosome replication and that of episomes such as F are controlled by similar processes. In other words, the relationship between the number of copies of F and the number of chromosome origins should remain constant at all times both under steady-state conditions and during transitions from one steady-state of growth to another. A direct demonstration

of the validity of this premise is thus necessary before the merits of any of the above models can be ascertained. Since current evidence is consistent with the hypothesis that the initiation mass of the chromosome remains constant over a range of cell growth rates (Cooper and Helmstetter 1968; Donachie 1968), it might be expected likewise, that F replication would occur at a constant mass over this range. This would preclude coupling between F replication and any phase of the chromosome replication cycle other than initiation. The data shown in figure 26 lend support to this notion but do not preclude the possibility that F replication is coupled to initiation or to the replication of genes located close to the origin.

One approach to analysing the relationship between F replication and the cell cycle has been the use of synchronous cultures. F replication is generally monitored by noting the time in the cell cycle at which the potential to synthesise episomally specified β-galactosidase doubles. Some early experiments of Nishi and Horiuchi (1966) using E. coli K_{12} synchronised by the filtration method of Maruyama and Yanagita (1956) and growing in glycerol minimal medium suggested that F replication in this medium occurred rather early in the cell cycle. The validity of this conclusion is questionable since under their conditions, neither β-galactosidase nor D-serine deaminase potential in the F^- parent, nor D-serine deaminase in the F'lac

derivative increased in the expected stepwise fashion. The possibility that artefacts were introduced by the synchronisation procedure therefore cannot be excluded. Donachie and Masters (1966) however have also concluded that F replication occurs early in the cell cycle. Using a sucrose gradient centrifugation procedure to obtain synchronous cells (Mitchison and Vincent 1965), they found two stepwise increases in β-galactosidase potential during the cell cycle in an Flac/*lac*$^+$ strain whereas in the F$^-$ parent under the same conditions, they could detect only a single step. This additional step in potential occurred early in the cell cycle.

Experiments using the membrane elution technique (Helmstetter and Cummings 1963, 1964) have yielded data which have been interpreted in different ways. Zeuthen and Pato (1971), using an *E. coli* B/rΔ*lac*/Flac strain, concluded contrary to the findings of Donachie and Masters (1966), that F replication occurs late in the cell cycle over a range of growth rates. Their data however can be interpreted in a number of ways. The scatter of the data is such that they are consistent with F replication occurring coincident with termination of a round of chromosome replication in all but fast growing cells (τ = 24 minutes). Equally, as argued by Davis and Helmstetter (1973), the data are consistent with the idea that F replication occurs at a similar cell age to that at which chromosome initiation takes place but that in cells growing with

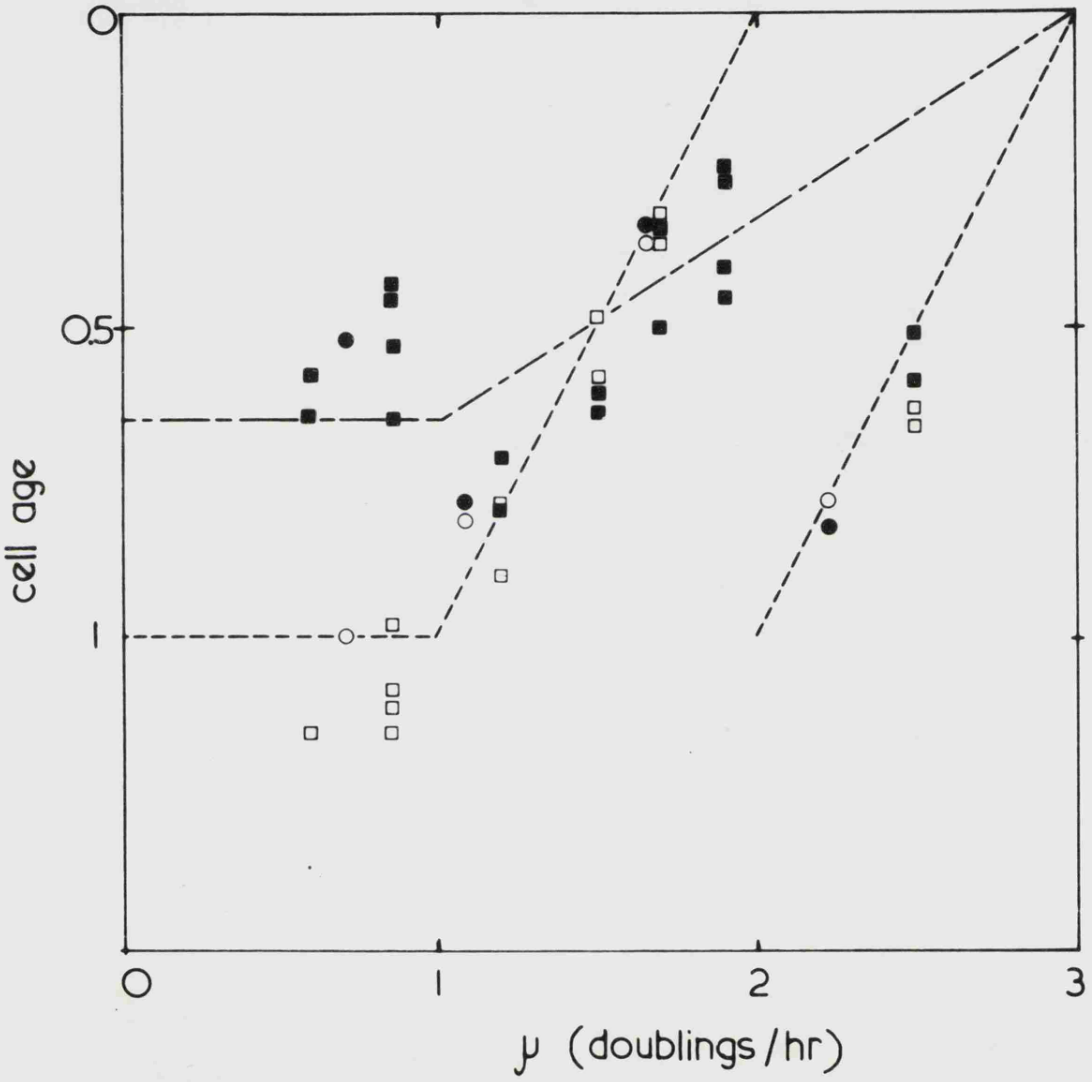
doubling times greater than 50 minutes, F replication occurs significantly earlier. Data from a similar series of experiments by Davis and Helmstetter (1973) have been interpreted to show that F replication in cells growing with generation times of less than 55 minutes occurs at approximately the same time as initiation of chromosome replication, and again, in cells growing with doubling times greater than this, it occurs significantly earlier. Both sets of data are shown in figure 32 and, as can be seen, are in good agreement.

Cooper (1972) has investigated the pattern of F replication in a similar way. He has used the membrane elution technique, but in addition, has subjected the membrane bound cells to a shift-up in growth rate. Although the synchrony is poor, and thus his data rather difficult to analyse, he has concluded that the variation in cell age at which F replication occurs during the shift is similar to the variation in cell age at which chromosome initiation occurs. Again, in slow growing cells, F replication occurs earlier than initiation of chromosome replication. He also concludes that F replication occurs progressively earlier in the cell cycle than chromosome initiation as the growth rate is decreased. In other words, the initiation mass for F replication increases progressively with growth rate. In regard to experiments of this nature, it should be noted that the elution technique becomes less accurate at both ends of the growth rate range. One conclusion which can be drawn from all three sets

FIGURE 32.

TIMING OF F REPLICATION IN THE CELL CYCLE.

———, theoretical time in the cell cycle at which new rounds of replication occur; ——— – ———; theoretical time in the cell cycle at which termination of rounds of replication occurs. The data are taken from Zeuthen and Pato (1971) (■ , □) and from Davis and Helmstetter (1973) (•,o). The closed symbols represent the estimated time in the cell cycle at which F replicates, as judged by the doubling in episomally specified β-galactosidase. The open symbols represent the estimated time in the cell cycle at which chromosome initiation occurs.


0
0.5
1
cell age
0
1
2
3
μ (doublings/hr)

of data however, is that the cell age at which F replication occurs changes in a manner similar (but not necessarily identical) to chromosome initiation.

A second approach to determining the timing of F replication in relation to chromosome initiation has been to measure the number of copies of F relative to other cell parameters in steady state batch cultures. Frame and Bishop (1971) using an RNA:DNA hybridization procedure, have estimated that F'8(gal) DNA in cells growing with a doubling time of between 50 and 55 minutes, comprises some 4% of total DNA. This is equivalent to approximately 1.4 copies per genome equivalent of chromosomal DNA or, assuming C+D to be 70 minutes, and τ = 50 minutes, 0.8 copies per chromosome origin. This value is in good agreement with the estimates made by Collins and Pritchard (1973) of the number of copies of an F'lac particle per chromosome origin in cells growing at a comparable rate. Using the measured contour length to estimate the molecular weight of the episome and a DNA:DNA hybridization procedure to estimate the percentage of F DNA in total DNA, they found that the number of copies of the sex factor per chromosome origin decreased from 0.9 in cells growing with a doubling time of about 60 minutes to 0.5 in cells growing with a doubling time of about 22 minutes. This finding is not consistent with the view that F replication occurs at a constant cell mass over a range of growth rates and is qualitatively

in agreement with the results of Cooper (1972).

In addition to estimating the number of copies of F'8(gal) by hybridization, Frame and Bishop (1971) were able to show that the activity of galactokinase and galactose-I-phosphate uridyl transferase in cells with and without episomal and chromosomal gal genes was proportional to the number of gal genes present if the gratuitous inducer D-fucose was used. If the observation of proportionality between gene dosage (or more strictly, gene concentration) and gene output is valid in the case of other enzyme systems in which it is possible to control externally the degree of repression or derepression operating and if this is also true over a range of growth rates, then, as discussed in sections 4.I and 4.II, measurements of enzyme activity in haploid and merodiploid strains might prove useful for investigating not only F replication but the replication of other bacterial plasmids.

It has been noted that the introduction of an F prime factor does not necessarily give rise to a twofold increase in enzyme activity in the resulting merodiploid strain. In some cases the increase is less, and in others more than twofold. This has been attributed to a change in the transcription and/or translation frequency of the gene when located on the episome (Stetson and Somerville 1971). Even if the rate of enzyme synthesis were independant of the location of the gene, (with

respect to episome or chromosome) one would not necessarily expect to observe a twofold increase in the merodiploid strain. The magnitude of the increase will depend on several factors. In the first place, one would not expect proportionality between gene concentration and gene output if the gene in question were regulated by internal effectors (see section 4.IV), and secondly, the magnitude of the increase will depend on the concentration of the episomal gene relative to the concentration of the chromosomal gene. This in turn will be dependant on the growth rate of the cells (and the replication velocity), the location of the gene on the chromosome and the mode of F replication. With this in mind, it may be of interest to analyse several sets of published data concerning the expression of genes in merodiploid strains. I will consider only enzymes which are inducible or which are produced at basal level or constitutive levels. It is assumed throughout that:- a) enzyme output in these situations is proportional to gene concentration and does not depend on the location of the gene with respect to episome or chromosome, b) the chromosome origin is located at 67 or 73 minutes on the E.coli linkage map (Taylor and Trotter 1972). Calculations based on the latter value are placed in brackets, c) that replication is bidirectional and symmetric and, d) that the transit time of a replication fork is 45 minutes.

Cassio et.al. (1970) have reported that E.coli K_{12} carrying the episome F'32(dsd$^+$) and growing in nutrient broth shows an increased basal level of D-serine deaminase of 1.77 fold compared to the isogenic female strain. If it is assumed that both the male and female strains grow at the same rate with a doubling time of about 25 minutes, calculation shows that the number of copies of F32 compared to the chromosomal dsd locus is 0.77 (0.76) and the number of copies per chromosome origin is 0.41 (0.35). In glucose minimal medium, the male was found to have basal D-serine deaminase levels 2.0 fold higher than the female. If a generation time of 60 minutes is assumed for E.coli K_{12} growing in this medium at 37°C, then the ratio of F32 to chromosomal dsd loci is 1.05 (1.03) and the number per chromosome origin is 0.8 (0.75). These results are qualitatively in agreement with those of Collins and Pritchard (1973).

In a paper by Garen and Garen (1963) concerned with complementation between structural mutants of alkaline phosphatase, it was shown that for E.coli K_{12} growing in L.broth, the activity of alkaline phosphatase in a F'pho/pho$^+$ merodiploid strain was 2.4 fold higher than in the isogenic female. In this case the proportion of F^- segregants was shown to be less than 5%. If we assume that in L.broth, the cells grow with a generation time of about 22 minutes, then the number of copies of the F prime compared to the

chromosomal phosphatase structural gene is 1.4 (1.4) and the ratio of F' to chromosome origins is 0.49 (0.6).

Several estimates have been made concerning the activity of β-galactosidase in haploid and merodiploid strains. Revel (1965) found that in cells growing in a glycerol amino acid medium with a doubling time of between 56 and 60 minutes (Revel et.al. 1961), there was approximately a 2.1 fold difference in the levels of fully induced β-galactosidase between haploid and merodiploid strains. This corresponds to a ratio of episomal to chromosomal lac genes of 1.1 (1.1) and a ratio of episome to chromosome origins of 0.76 (0.82). Both the enzyme and hybridization data have been plotted in figure 33. It can be seen that both types of data are in accord with notion that the number of copies of the episome per chromosome origin decreases with increasing growth rate. Qualitatively, it is the behaviour expected if initiation of chromosome replication occurs at a constant cell mass and F replication were coupled in some way to the surface area of the cell, or if it were directly coupled to the replication of chromosomal genes located some distance from the origin (section 4.I and II). The curve drawn in figure 33 shows the change in surface area:mass ratio with growth rate. The values are taken from table 5 and have been normalised to a value of 0.8 for cells growing with a mass doubling time of 60 minutes.

One way in which the notion of coupling of F replication to a

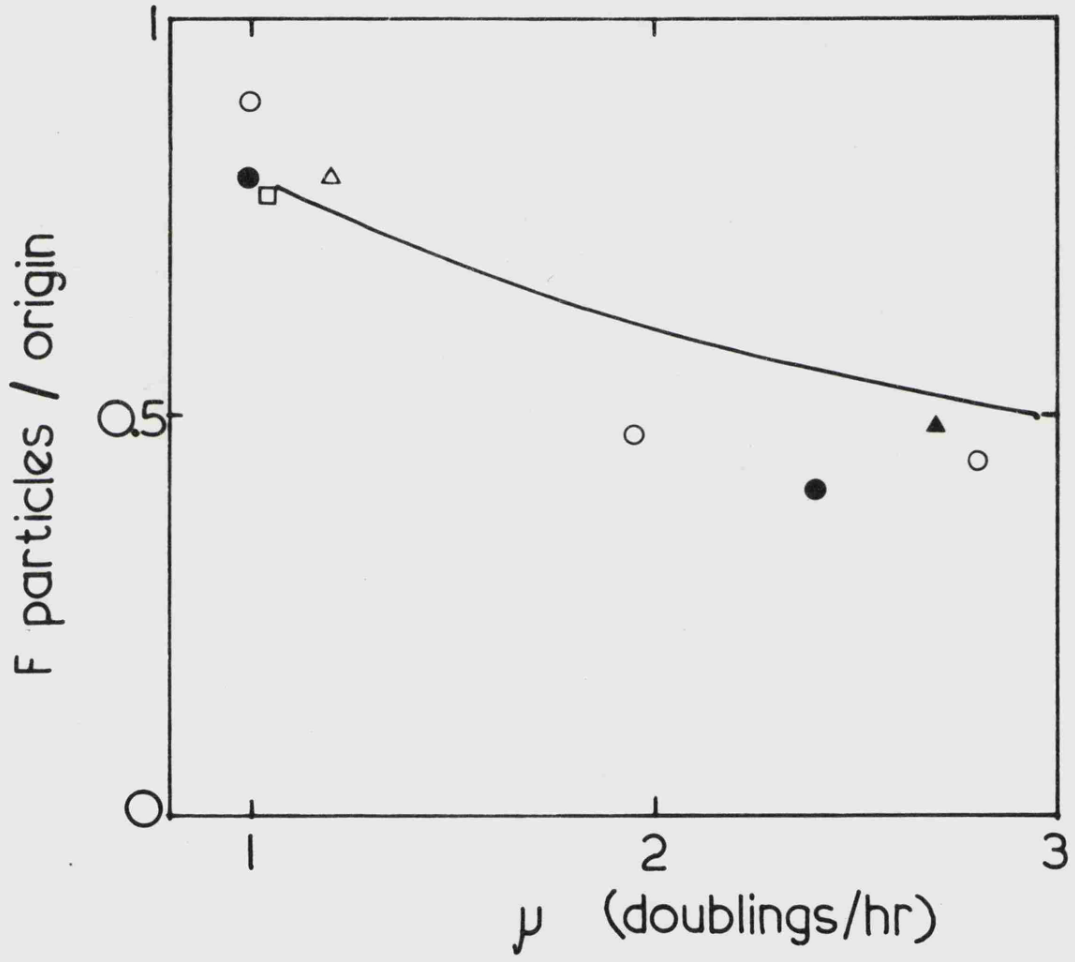
FIGURE 33.

VARIATION OF THE NUMBER OF COPIES OFF PER CHROMOSOME ORIGIN WITH GROWTH RATE.

The methods used to calculate the number of F particles per origin are discussed in the text. The curve shown is the theoretical change in the surface area:mass ratio (Schaechter et.al. 1958).

Data are taken from:

Collins and Pritchard (1973)	(o)
Revel (1965)	(□)
Frame and Bishop (1971)	(Δ)
Cassio et.al. (1970)	(●)
Garen and Garen (1963)	(▲)


1
0.5
0
F particles / origin
1
2
3
μ (doublings/hr)

constant surface area might be tested is to use the observed size changes in *thy*⁻ cells with thymine concentration as a means of changing the surface area:mass ratio in a manner independant of growth rate. In practice however, as noted in section 4.II, it is difficult to quantitate the change in surface area. Consequently, the predicted change in F concentration on a surface area model cannot be estimated.

A further approach to the question of the control of F replication has been to observe the relationship between F and chromosome replication following treatments such as amino acid starvation (Bazaral and Helinsky 1970) or after shifting a temperature sensitive initiation mutant (*dnaA* or C) to the non-permissive temperature (Zeuthen and Pato 1971).

Bazaral and Helinsky (1970) have concluded that, following amino acid starvation of *E.coli* K_{12} CR34 F^+, chromosomal and F synthesis remain in proportion. They suggest that the initiation of F factor synthesis is dependant on chromosome replication through a mechanism which does not depend on protein synthesis.

The results obtained using a temperature sensitive initiation mutant (Zeuthen and Pato 1971) are concordant with those obtained with amino acid starvation. Following a shift to the non-permissive temperature, F replication (as judged by the capacity to synthesise episomally specified β-galactosidase) continues until chromosomal DNA synthesis ceases. Episome replication resumes soon after

returning the culture to the permissive temperature.

To summarise then, it would seem, on present evidence, that F replication requires chromosomal DNA synthesis. This does not seem to be a specific requirement for chromosome initiation, since F replication continues for some time after chromosome initiation has been inhibited. Nor does it seem to be a requirement for termination since, on returning a dnaC/Flac culture from the non-permissive to the permissive temperature, F replication proceeds almost immediately. The cell age at which F replication occurs has been shown to vary with growth rate, and may or may not coincide with initiation of chromosome replication. Data concerning this point is at present inconclusive. Experiments utilizing synchronous cultures can be interpreted to show that the relationship between F replication and initiation of chromosome replication is invariant with growth rate. On the other hand, several sets of data, both direct and indirect, from experiments with steady-state batch cultures suggest that the initiation mass for F replication may increase with growth rate. This would be the behaviour expected if F replication were coupled directly to the replication of chromosomal genes located some distance from the origin. The data presented in section 4.II precludes such a possibility, but does not preclude coupling to chromosome initiation or to replication of genes located close to the chromosome origin. Unfortunately, technical

difficulties have prevented the utilization of the observed cell size changes (figure 17) with thymine concentration as a means of investigating the notion that F replication is coupled to a constant surface area. Until the relationship between chromosome initiation and F replication has been more completely documented, it would seem premature to discuss the behaviour of F with regard to the proposed models described at the beginning of this section.

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SUMMARY

Previous work in this laboratory has indicated that in thymine low requiring strains of Escherichia coli, the transit time of a replication fork can be varied without an appreciable effect on the growth rate, simply by varying the thymine concentration in the growth medium. This results in a change in the DNA concentration and therefore changes the dosage of most genes. I have defined three more precise parameters which have previously been encompassed in the term gene dosage. These I have called gene concentration, relative gene dosage, and gene dosage. On theoretical grounds, these three parameters change in separate and distinct ways as a function of the replication velocity and as a function of the relative position of the gene on the chromosome. In order to distinguish the relative importance of each of them in determining the overall rate of protein synthesis, I have compared the rate of enzyme synthesis from five genes in a fully repressed or induced state and one in an autogenous state in cultures growing at the same rate but having different transit times.

The data permit the following conclusions:

(1) The initiation mass is independent of the transit time of a replication fork at one growth rate.

(2) Replication is bidirectional. The chromosome origin is near the gene coding for tryptophanase and the terminus near the gene specifying tryptophan synthetase. The data place the origin in the region of 67 minutes on the E.coli linkage map.

(3) The output of a gene under conditions when it is subject to regulation by a cytoplasmic effector decreases as its concentration is increased.

In addition, I have used the observed differential effect of changes in replication velocity on gene concentration to determine if the replication of the sex factor F is directly coupled to any stage of the chromosome replication cycle. The data preclude the possibility that F replication is coupled to any part of the chromosome replication cycle other than to initiation (or to the replication of genes close to the origin) or, at the growth rate used here, that it occurs at a constant cell mass.