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Review Lecture: On the Growth and Form of a Bacterial Cell

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REVIEW LECTURE

ON THE GROWTH AND FORM OF A BACTERIAL CELL

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'In this work I shall show that, contrary to the orthodox teaching, the cells of bacteria are constantly changing in size and form structure; but that instead of being phases in a rather vague and complex life-cycle, they occur with great regularity and are governed by relatively simple laws which, after more data have been accumulated and analysed, may probably be very precisely formulated.' Henrici (1928)

CONTENTS

The size, shape and composition of cells in cultures of bacteria maintained in steady states of exponential growth depend on the cultural conditions employed. Important factors influencing these parameters are the growth rate of the culture and the transit time of replication forks from one end of a chromosome to the other. The considerable progress which has been made in the last ten years in elucidating the rules governing the form and composition of cells of *Escherichia coli* as a function of growth rate and transit time is outlined in the Review.

Introduction

A significant advance in our understanding of the rules governing the size and composition of bacterial cells was made by Schaechter, Maaløe & Kjeldgaard in 1958. They made a number of measurements on cultures of *Salmonella typhimurium* which were effectively in steady states of exponential growth at one temperature in a variety of culture media of increasing richness which supported progressively increasing growth rates. What they observed is summarized diagrammatically in figure 1. Average cell mass increased exponentially with increasing growth rate; the average amount of RNA per cell also increased exponentially, but at a somewhat faster rate so that its contribution to total mass increased as the growth rate increased; the average amount of DNA per cell also increased but at a slower rate than cell mass so that the DNA to mass ratio (the DNA concentration) decreased.

The importance of these experiments was threefold. They showed that the significant variable affecting cell size and composition was the growth rate, the parameters measured being relatively insensitive to the composition of the growth medium. They emphasized the value of making comparisons between cultures which were growing under comparable and definable conditions, and they drew the attention, especially of those who were not bacteriologists, to the fact that the variation in cell size and composition which accompanied changes in growth rate followed apparently simple rules which should therefore be open to elucidation.

I propose to review some of the progress made since 1958 towards an understanding of the cellular mechanisms underlying these changes in composition in *Escherichia coli* and *S. typhimurium*. I shall be primarily concerned with the determination of DNA concentration, cell size, and a third variable not shown in figure 1 – cell shape.

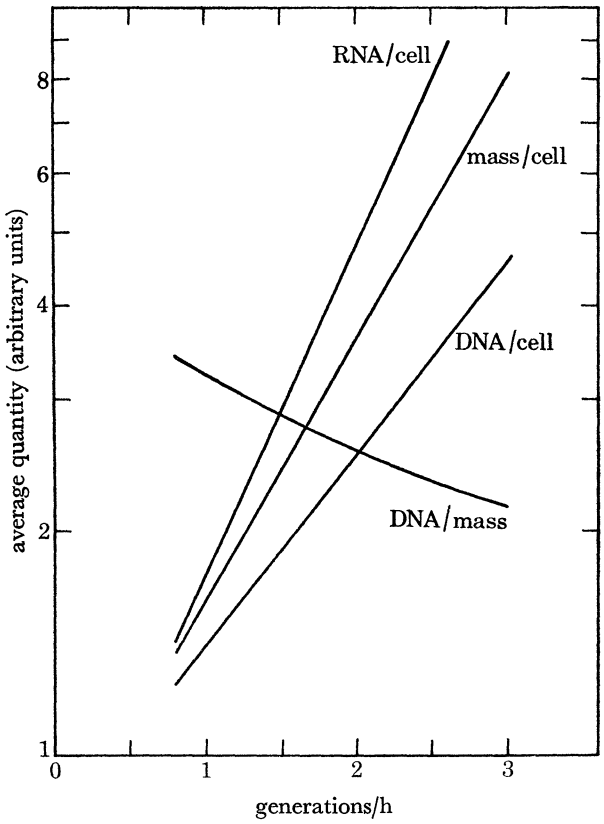
DNA concentration

The change in DNA concentration which is associated with a change in growth rate is now known to be a consequence of the way in which the rate of DNA synthesis in a cell population is matched to the rate of increase of the culture mass. I shall therefore begin with a description of the mechanism involved.

(*a*) *Control of chromosome replication*

The chromosome of *E. coli* consists of a ring of double-stranded DNA. Rounds of replication are initiated at a unique site on this ring called the origin of replication, or chromosome origin. The location of this origin on the linkage map has not been precisely determined but can be placed with some confidence between minutes 64 and 74 on the time of entry map (figure 2*a*) of this species (Masters & Broda 1971; Bird, Louarn, Martuscelli & Caro 1972). Unpublished data from this laboratory (Chandler & Pritchard, 1974) place it at minute 67. Following initiation, chain elongation proceeds in both directions from the origin until the replication forks meet at a position which appears to be half way round the chromosome from the origin (figure 2*b*). This position is loosely described as the chromosome terminus, although there is no reason at present to believe that it is other than the point at which converging replication forks meet. The time taken for a replication fork to traverse half the chromosome is conventionally designated *C* (Cooper & Helmstetter 1968). It is generally assumed that forks travel at constant velocity although there is no unambiguous evidence bearing on this question.

There are thus two distinct processes, which can be distinguished experimentally, which



RE 1. Average composition of steady-state exponential cultures of *S. typhimurium* as a function of growth rate. The curves are drawn from the empirical equations given by Maaløe & Kjeldgaard (1966).

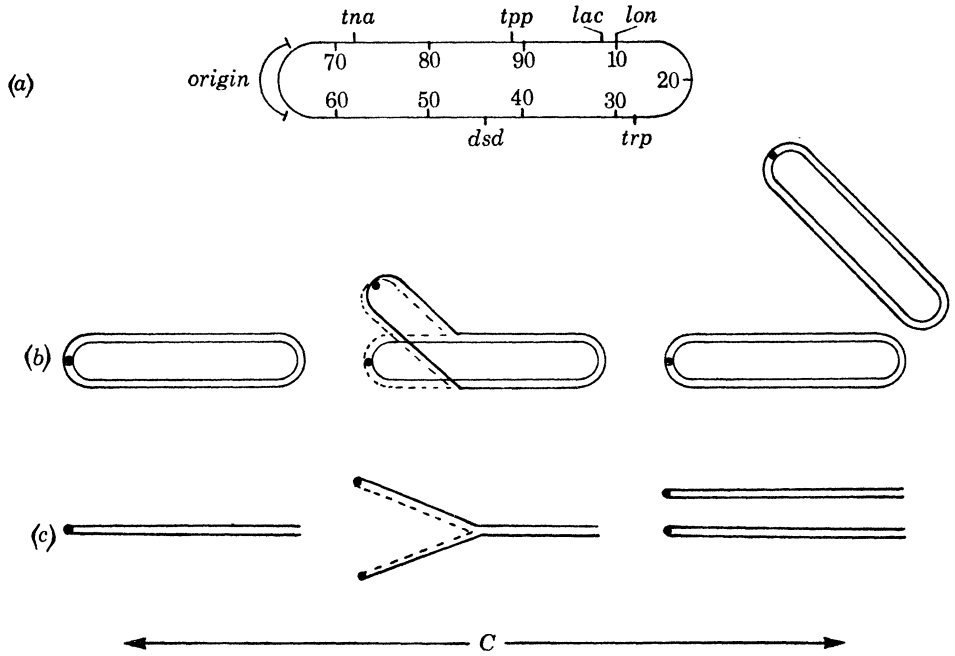


FIGURE 2. (*a*) Genetic map of the chromosome of *E. coli* showing the location of genes referred to in this review. Numbers inside the ring refer to minutes on the time of entry map. (*b*) Diagrammatic representation of bidirectional replication of a circular chromosome. (*c*) Corresponding linear representation of a replicating half chromosome used in subsequent figures.

could be involved in determining the rate of DNA synthesis. One is the frequency of initiation of new rounds; the other is the rate of addition of deoxynucleotides to growing polynucleotide chains.

It might be supposed that an increase in the rate of DNA synthesis in response to an increase in growth rate could be achieved by an increase in the frequency of initiation of new rounds of replication, by a reduction in the transit time of replication forks, or a combination of the two. In fact, it has been clear since 1964 (Oishi, Yoshikawa & Sueoka 1964; Pritchard, 1965; see also Maaløe 1961) that a change from one steady state rate of DNA synthesis to another is achieved solely by changing the frequency of initiation of rounds of replication, although this important point did not become widely appreciated until 1968 when Cooper & Helmstetter demonstrated that the transit time of replication forks in one strain of *E. coli* was nearly constant in cultures with doubling times ranging from about 70 to 20 min† leaving the frequency of initiation as the only variable.

The fact that the rate of DNA synthesis is determined by the frequency of initiation of new rounds of replication and independent of the transit time of replication forks is so important for an understanding of the remainder of this Review that a simple analogy seems in order. We can imagine the chromosome as a road. Motor cars join the road at a constant frequency (initiation) and travel along it at a constant velocity (replication) until they reach the end (termination). Under steady-state conditions the frequency with which cars arrive at the terminus will be equal to the frequency with which they join the road and independent of the velocity with which they travel. The velocity is not irrelevant however: it will determine the number of cars on the road at any one time. Thus if one car per hour enters the road and the journey takes 1 h there will always be one car somewhere on the road. If the journey takes 2 h or cars join the road every half an hour each car will have travelled only half the distance before being followed by a second. In terms of the bacterial chromosome this corresponds to multifork (or dichotomous) replication.

The reason why this rather obvious aspect of DNA replication was not appreciated earlier is that there is a limiting situation in which the rate of DNA synthesis *would* be determined by the velocity of replication. This is the case where not more than one replication fork can be present on each chromosome (or half chromosome in the case of bidirectional replication) at any one time. In terms of the analogy this would correspond to a situation in which there was only one car available. The frequency with which this car could join the road would depend on how long it spent on each journey. Until dichotomous replication was discovered (Oishi *et al.* 1964; Pritchard & Lark 1964) this limiting case was implicitly assumed in all discussions of DNA replication in bacteria.

If the rate of DNA synthesis is determined by the frequency of initiation the bacterial cell must be able to monitor its growth rate and adjust this frequency so that new rounds of replication occur once, and only once, each time the culture mass doubles. At one level the nature of this biological clock is understood. New rounds of replication appear to be initiated at a constant mass-chromosome origin ratio which is independent of the growth rate in cultures with mass doubling times less than about 70 min (Donachie 1968; Pritchard 1968). Thus we can write

$$(m/n)_1 = k_1, \qquad (1)$$

† The relation between growth rate and C in cultures with doubling times greater than about 70 min is not clear: some data (Helmstetter, Cooper, Pierucci & Revelas 1968) indicate that at slow growth rates C increases in length in proportion to the increase in doubling time while other data (Kubitschek & Freedman 1971) suggest that the constancy of C is maintained even at very slow growth rates. None of the arguments in the body of this Review is affected by this uncertainty.

where m is the cell mass, n the number of chromosome origins present and k_1 is a constant which has been called the initiation mass (Donachie 1968)†. This relation constitutes a biological clock since when $m/n = k_1$ initiation will occur leading to a doubling in the number of chromosome origins. Initiation will consequently not occur again until the culture mass has doubled restoring the mass to chromosome origin ratio to k_1.

Possible mechanisms whereby the cell might titrate its mass to chromosome origin ratio have been discussed elsewhere (Helmstetter *et al.* 1968; Marvin 1968; Pritchard 1968; Pritchard, Barth & Collins 1969; Sompayrac & Maaløe 1973).

(*b*) *DNA concentration and growth rate*

If the initiation mass is constant it can be shown that the average DNA concentration in a culture which is in a steady state of exponential growth will be given by the following expression (Pritchard & Zaritsky 1970):

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

where $\bar{G}$ is the average amount of DNA per cell in chromosome equivalents, $\bar{M}$ is the average cell mass, τ is the doubling time and k is a constant. If C is constant then $\bar{G}/\bar{M}$ will fall progressively as the growth rate is increased as shown in figure 1.

Using synchronous cultures of a strain of *E. coli* called B/r Cooper & Helmstetter (1968) showed that C had a value of 40 to 45 min in cultures with doubling times less than about 70 min. The rate of fall of $\bar{G}/\bar{M}$ with increasing growth rate which can be calculated from the data of Schaechter *et al.* (1958) in *S. typhimurium* is in good agreement with equation (2) if a constant value of C of 45 min is assumed in this species also.

The reason for the fall in DNA concentration with growth rate is illustrated diagrammatically in figure 3. In this figure one unit mass of cytoplasm containing one chromosome is shown increasing exponentially with time. The fact that each time its mass doubles the cell will divide in two can be ignored. At time zero in figure 3*a* the chromosome will embark on a round of replication since $m/n = k_1$. The horizontal bars represent the time taken (assumed here to be 40 min) for a replication fork to transverse the chromosome. Since in figure 3*a* the mass doubles every 70 min there will be a gap in time between termination of one round of replication and commencement of the next. By the time the culture mass has increased from 1.0 to 3.0 it will contain four fully replicated chromosomes.

If, at time zero, the same cell had been dropped into a richer medium permitting a doubling time of 40 min (figure 3*b*) a new round of replication would start immediately the preceding one was completed. There would be no gap between rounds of replication. More important, by the time the culture mass had increased to 3 units the second round of replication would not have been completed and there would be two half replicated chromosomes present instead of four fully replicated chromosomes – the DNA concentration would have fallen.

If the cell was dropped into an even richer medium permitting a doubling time of 30 min, a second round of replication would commence before the first was completed. Replication would now be dichotomous. Moreover, when the culture mass reached 3 there would be a single chromosome with three replication forks present (figure 3*c*) – the DNA concentration would have fallen even further.

† Whether the initiation mass remains unchanged in cultures with doubling times greater than 70 min cannot be established until the uncertainty about C at these growth rates has been removed.

Several important features of this pattern of replication should be noted. First, during the transition from one growth rate to a faster one the rate of DNA synthesis would be less than the rate of mass increase, as was found experimentally by Kjeldgaard, Maaløe & Schaechter (1958) in steady-state exponential cultures which had been 'shifted-up' from a poor to a rich growth medium, but once this transitional period had elapsed the rates of DNA synthesis and

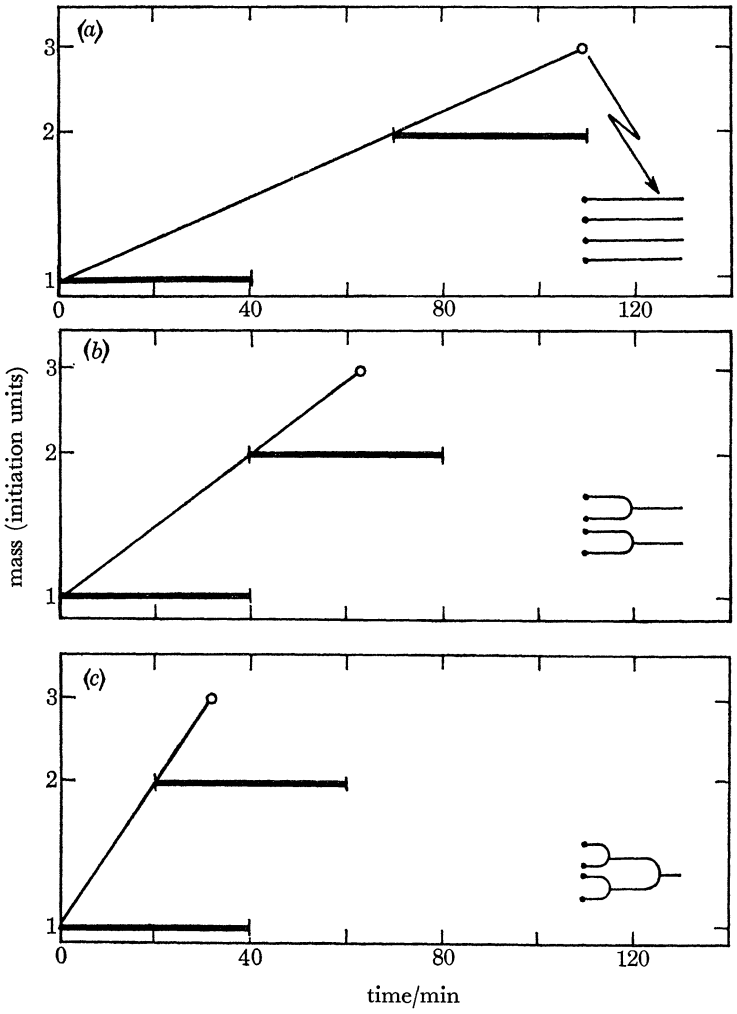


FIGURE 3. The effect of different growth rates on DNA concentration and on the configuration of an 'average' chromosome. The transit time of a replication fork is denoted by thick horizontal bars and is assumed to be 40 min. The diagram in the bottom right-hand corner of each panel shows the chromosome configuration that would be found in a culture mass 3 × the initiation mass.

mass increase would be identical since, as described earlier, the rate of DNA synthesis is determined by the frequency of initiation of new rounds of replication. Thus when the culture mass reached 6 the culture shown in figure 3*a* would have eight complete chromosomes and that shown in figure 3*b* would have four replicating chromosomes.

Secondly, it should be noted that although the DNA concentration falls with increasing growth rate different genes are affected differently. The greatest reduction in concentration will be suffered by genes located at the chromosome terminus. For genes located at the chromosome

origin there will be no change at all in concentration. This important point has often been misunderstood, it being assumed that dichotomous replication implies an *increase* in the number of genes located near the chromosome origin. There is of course an increase in their *relative dosage* (gene: DNA ratio) but, as I shall demonstrate later, it is gene concentration not relative gene dosage which is the important factor, other than regulation, influencing gene output. This differential effect of growth rate on gene concentration provides a rational explanation for non-random gene order (apart from the well-known cases of contiguity of genes constituting an operon) in bacteria. We might anticipate that genes whose products subsume a major fraction of the cell's biosynthetic resources and which might therefore be undergoing transcription at a rate near to the maximum attainable at a particular growth rate would be located close to the chromosome origin. In this location the number of copies of the gene per unit mass would be as high as possible (and the concentration of gene product for a given rate of transcription therefore the maximum attainable) and would remain so at all growth rates. It is therefore perhaps significant that there appear to be six copies of the gene cluster coding for 5S, 16S and 23S ribosomal RNA (which account for less than 0.5 %, of the genome but are responsible for more than half of the instantaneous rate of RNA synthesis – see Nierlich 1972) and that although the map locations of only three of these clusters has been determined (see Jarry & Rosset 1973) 80 % of this RNA is transcribed from a small segment of the chromosome between map positions 54 and 75 spanning the chromosome origin (Unger, Birnbaum, Kaplan & Pfister 1972). Similarly the majority of the genes coding for ribosomal proteins, which likewise pre-empt a large fraction of the biosynthetic resources of the cell (Schlief 1967) also appear to be located in a single gene cluster at map position 64 (see Takata 1972).

Finally, it should be noted that the use of one genome equivalent of DNA or one 'chromosome' as the datum against which rates of synthesis of macromolecules such as ribosomal RNA (rRNA) at different growth rates are conventionally compared can be misleading, since the number of copies of a particular gene per genome equivalent will depend on its map location and also be different at different growth rates. Fortunately, these differences are now quantifiable.

(*c*) *DNA concentration as a function of C*

We can restate the relation between DNA chain elongation and growth by saying that their rates are not coupled since the growth rate can be altered without affecting the transit time of replication forks. This leads to the interesting possibility that it might be possible to alter the transit time of replication forks without affecting the growth rate.

To test this possibility it would be necessary to find some way of manipulating the rate of DNA chain elongation, and it occurred to us some years ago that there is a simple way in which this might be achieved. Thymine, so far as we know, has no other essential role in *E. coli* except as a constituent of DNA. Since thymine-requiring mutants can be readily isolated it might be possible to reduce the intracellular concentration of thymidine triphosphate (the immediate precursor of DNA thymidylate) in such mutants simply by adjusting the concentration of thymine in the growth medium so that it became the rate limiting factor in chain elongation.

The possibility that replication velocity might be altered in *thy*$^-$ strains without affecting growth rate also has extremely important technical implications since it is quite possible that even the concentrations of thymine routinely added to the growth medium used to cultivate such strains

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is associated with reduced rates of chain elongation. To see why this is so, one need simply recall that the standard method used to determine how much of a growth factor to add to the growth medium of a nutritional mutant is to add increasing concentrations until the growth rate and growth yield are no longer improved. Clearly this procedure might be inappropriate in the case of thymineless mutants since a concentration of thymine might be chosen which was not rate or yield limiting for growth but which nevertheless was rate limiting for chain elongation.

Using a variety of techniques we have been able to demonstrate that the rate of chain elongation can be more than halved in *thy*$^-$ strains by reducing the concentration of thymine in the growth medium and that this reduction in replication velocity does not lead to a detectable change in growth rate (Pritchard & Zaritsky 1970; Zaritsky & Pritchard 1971, 1973). In addition, our data show that concentrations of thymine that have been generally employed in growth media are rate limiting for chain elongation in several widely used *thy*$^-$ strains.

Two of the methods we have employed to detect a change in C are particularly instructive and will be described briefly here. One is to determine the effect of a change in thymine concentration on the DNA:mass ratio in cultures which are in steady states of exponential growth. The other is to determine the increment in DNA following inhibition of protein synthesis in such cultures growing in different thymine concentrations.

Figure 4 shows the results of an experiment of the first type. A culture which had been maintained in a steady state of exponential growth on one concentration of thymine (0.4 μg/ml) was diluted into fresh prewarmed medium containing a higher concentration (5 μg/ml). Notice that the rate of growth (rate of increase in absorbance) is not even transiently altered indicating that the lower concentration of thymine is not rate-limiting for growth. On the other hand, there is an immediate acceleration in the rate of DNA synthesis which subsequently falls back to the initial rate. The two cultures now have different DNA concentrations.

We interpret the growth pattern in figure 4 as follows. The low concentration of thymine in the starting culture is rate-limiting for chain elongation but not for mass increase. When the concentration of thymine is increased there is an immediate acceleration in the rate of chain elongation and therefore an increase in the rate of DNA synthesis. When the distribution of replication forks equilibrates to that appropriate to the new replication velocity the rate of DNA synthesis returns to the original steady-state rate since this is determined by the frequency of initiation of new rounds of replication which, in turn, is determined by the rate of mass increase which has not altered. The DNA concentration is permanently altered however, because the distribution of replication forks is different in the two cultures.

This experiment illustrates very clearly the danger of determining the appropriate concentration of thymine to add to the growth medium of a *thy*$^-$ strain simply by measuring the growth rate.

The magnitude of the difference in C between the two concentrations of thymine can be calculated from the difference in DNA concentration before and after the transition (using equation (2)) and also from the initial acceleration in the rate of DNA synthesis after the transition (Pritchard & Zaritsky 1970). Both estimates indicate an approximately twofold change in C between the two concentrations but both calculations assume that there is no effect of thymine concentration on the initiation mass.

The second method for detecting a change in C and estimating its magnitude depends only on the well-known observation that when protein synthesis is inhibited (by removal of a required amino acid from the growth medium for example) initiation of new rounds of replica-

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tion is blocked, but termination of rounds of replication in progress is completed. The increment in total DNA by a culture in a steady state of exponential growth under these conditions will depend on the distribution of replication forks and, as can readily be seen from figure 3, this will be different at different growth rates. It will also be different for different values of C. The predicted increment (ΔG) can be expressed quantitatively (Sueoka & Yoshikawa 1965) as

$$\Delta G = \frac{2^n n \ln 2}{2^n - 1} - 1 \qquad (3)$$

where $n = C/\tau$.

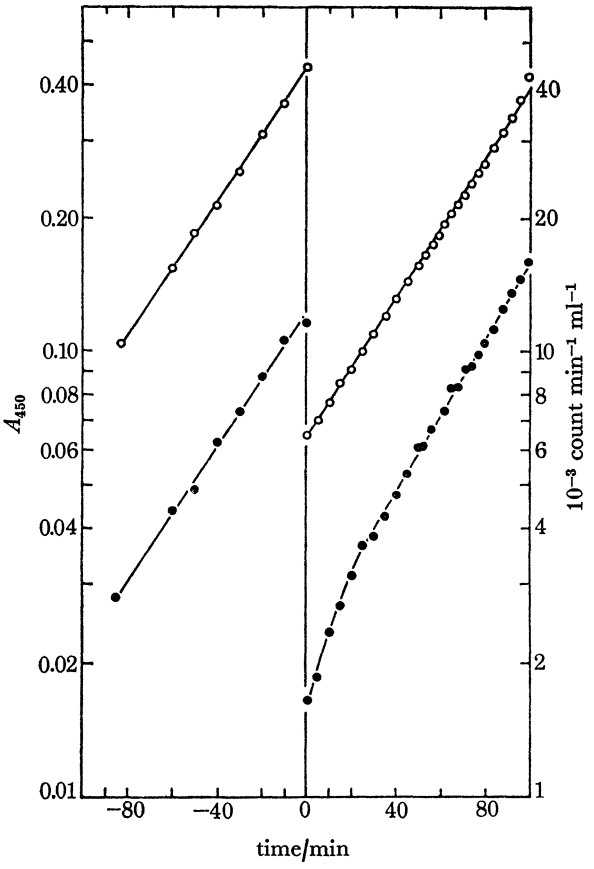

FIGURE 4. Effect of an increase in thymine concentration on DNA concentration. A steady-state exponential culture of *E. coli* $15T^-$ in glucose synthetic medium containing 0.5 μg/ml [^{14}C]thymine was diluted into identical medium containing 5.0 μg/ml [^{14}C]thymine at the same specific activity. Absorbance (○) and total DNA (●) were determined before and after the transition. (Reproduced from Pritchard & Zaritsky (1970).)

In one experiment (Pritchard & Zaritsky 1970), using the same strain as that used in the experiment shown in figure 4, we found that ΔG increased progressively from 61 % in a culture growing in medium containing 10 μg/ml to 130 % in a parallel culture containing 0.25 μg/ml. From this difference it can again be calculated that there is a greater than twofold change in C between the two extreme concentrations of thymine used. This method does not depend on any assumptions about the effect of thymine concentration on the initiation mass.

It is perhaps worth noting here that it has been frequently assumed (despite the initial observations and predictions of Maaløe & Hanawalt (1961)) that the increment in DNA will always be about 40 % when a bacterial culture is deprived of a required amino acid. As can be

seen from equation (3) the increment expected will depend on both the growth rate and the chromosome replication time in the culture and the predicted value is only 40 % when $C = \tau$. Our discovery that C is affected by the thymine concentration in the growth medium explains why highly variable increments can be found in the literature even when the same *thy*$^-$ strain and an identical growth medium has been used: each laboratory has its own favourite thymine concentration for addition to the growth medium.

The data in figure 4 also show that the growth rate of *E. coli* is not limited by the DNA concentration since a reduction of at least 40 % is compatible with no reduction in growth rate. Under normal conditions the majority of genes must either be transcribed at less than the maximal rate attainable (i.e. they must be under some degree of repression) or most gene products must be synthesized at a rate in excess of that needed to support the growth rate found. These arguments do not of course apply to genes located near to the chromosome origin.

One final point that should be made is that the value of C in *thy*$^+$ strains (and the minimal C obtainable in *thy*$^-$ strains) probably lies between 45 and 50 min in a variety of strains of *E. coli* and also in *S. typhimurium* (Spratt & Rowbury 1971).

Thymine incorporation in *E. coli*

(*a*) *Pathways*

It will not have escaped the notice of those who work with *thy*$^-$ mutants of *E. coli* that to double the replication time of the chromosome of the strain used in the experiments just described we used thymine concentrations as low as 0.25 μg/ml which is less than one-tenth of the concentration which is usually employed in cultivating such strains. It must therefore be emphasized that this well-known and widely used strain, which is a descendant of the first *thy*$^-$ strain ever isolated in *E. coli* (see Roepke 1967), is quite atypical in that it will grow at maximum rate on extremely low thymine concentration (0.25 μg/ml). Most *thy*$^-$ strains require tenfold higher concentrations and a more typical relation between thymine concentration and C is shown in figure 5 for another widely used strain (*E. coli* K 12 CR 34). The reason for the differences in thymine requirement of different strains is now largely understood, and I propose to consider now those features of thymine metabolism which are relevant to this Review (an exhaustive coverage of the literature pertaining to thymine metabolism can be found in O'Donovan & Neuhard (1970)).

Thy$^+$ strains of *E. coli* cannot use thymine as a source of thymidylate and cannot therefore incorporate thymine present in the growth medium into their DNA. *Thy*$^-$ mutants, which lack thymidylate synthetase and so have lost the ability to synthesise thymidylate, simultaneously gain the ability to convert thymine in the growth medium into thymidylate and incorporate it into their DNA. *Thy*$^-$ mutants are thus pleiotropic. The basis of this pleiotropy is as follows. The first step in thymine (T) utilization is its conversion to thymidine (T-dRib) mainly (Beacham & Pritchard 1971) by the enzyme thymidine phosphorylase:

$$\text{T} + \text{P-1-dRib} \longleftrightarrow \text{T-dRib} + \text{P}_\text{i}.$$

This is followed by phosphorylation of thymidine at the 5′ position by thymidine kinase to give thymidine-5-phosphate (T-dRib-P):

$$\text{T-dRib} + \text{ATP} \longleftrightarrow \text{T-dRib-P} + \text{ADP}.$$

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The thymidine monophosphate is then successively converted to thymidine diphosphate and triphosphate which is the immediate precursor of DNA thymidylate.

The reason why wild-type cells cannot use thymine is simply that they have a negligible pool of deoxyribose-1-phosphate (P-1-dRib) and therefore cannot convert significant amounts of thymine to thymidine. In *thy* mutants the *de novo* synthesis of thymidylate from deoxyuridine-5-phosphate (U-dRib-P) by thymidylate synthetase is blocked

$$\text{U-dRib-P} \not\longrightarrow \text{T-dRib-P}.$$

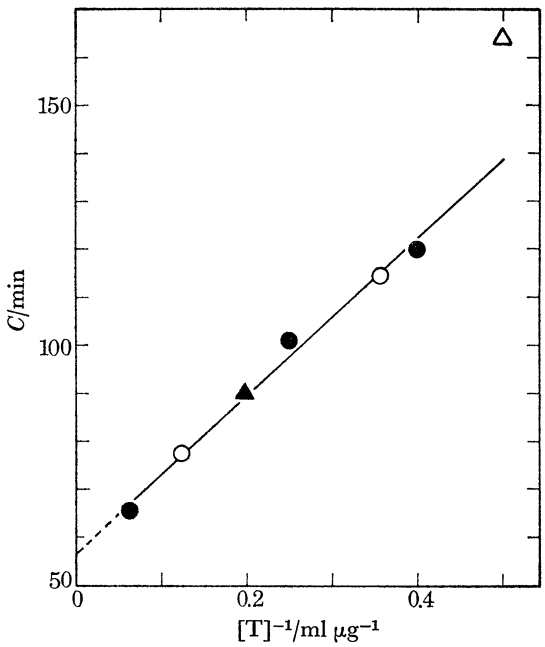

FIGURE 5. Replication time of the chromosome of *E. coli* CR 34 as a function of the concentration of thymine in the growth medium. Values calculated from changes in DNA concentration (●, △, ▲) in different experiments and from the increment in DNA after amino acid deprivation (○). (Reproduced from Zaritsky & Pritchard (1971).)

Deoxyuridine-5-phosphate accumulates and is degraded first to deoxyuridine (U-dRib) by an unknown phosphatase and then to P-1-dRib by thymidine phosphorylase

$$\text{U-dRib} + \text{P}_\text{i} \longleftarrow \text{P-1-dRib} + \text{U}.$$

Thus the lesion which prevents *de novo* synthesis of thymine fortunately and fortuitously provides the cell with the source of P-1-dRib which it requires to utilize thymine (see Beacham & Pritchard 1971).

It so happens that P-1-dRib is itself degraded by two inducible enzymes, phosphopentomutase and deoxyriboaldolase. It is for this reason that *thy*$^-$ mutants of *E. coli* typically require at least 20 μg/ml thymine in the growth medium in order to form colonies. Such high concentrations are necessary in order that synthesis of thymidine from P-1-dRib can compete significantly with its degradation.

Thy$^-$ strains give rise to secondary mutants capable of forming colonies on media containing about 2 μg/ml thymine. The majority of these strains have lesions in the genes specifying

deoxyriboaldolase or phosphopentomutase. The breakdown of P-1-dRib in these strains is consequently blocked and a larger pool maintained. Such mutants are often described as 'thymine-low-requirers' (Tlr.). The whole reaction scheme is summarized in figure 6.

The majority of *thy*$^-$ strains in common use are phenotypically Tlr. There is an additional and heterogeneous class of *thy*$^-$ strains which we have termed 'super-lows' which can form colonies on media containing as little as 0.2 μg/ml (Ahmad & Pritchard 1971). Two strains of this type which have been studied in detail have been shown to carry a third mutation, different in each case, which affects the regulation of the synthesis of thymidine phosphorylase and

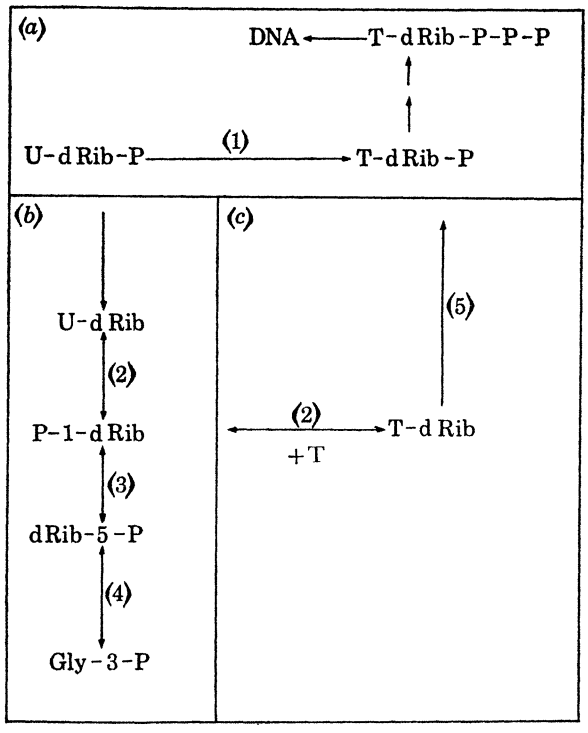

FIGURE 6. Metabolism of thymine derivatives. (*a*) *De novo* pathway for synthesis of thymidine triphosphate. (*b*) Catabolism of deoxyuridylate to provide a source of deoxyribose-1-phosphate which is used (*c*) to synthesize thymidylate from thymine. (1) Thymidylate synthetase; (2) thymidine phosphorylase; (3) phosphopentomutase; (4) deoxyriboaldolase; (5) thymidine kinase.

probably results in an exceptionally high P-1-dRib pool. Details of the these mutations need not concern us here. As mentioned earlier, *E. coli* 15T$^-$ also is a super-low strain. The basis of the super-low property of this strain has not been determined but it is different from that in the two super-low strains mentioned above (Beacham, Beacham, Zaritsky & Pritchard 1971).

It should be emphasized that the enzymes thymidine phosphorylase, phosphopentomutase and deoxyriboaldolase are components of a degradative pathway which permits the cell to utilize deoxynucleosides as carbon sources. That their presence permits *thy*$^-$ strains to utilize thymine as a DNA precursor is a fortunate laboratory artefact. It is therefore perhaps not surprising that the relation between [1/T] and *C* shown in figure 5 resembles a simple saturation curve and suggests that the internal dTTP concentration is determined primarily by the product of the external concentration of thymine and the internal concentration of P-1-dRib.

(*b*) *Pools*

If our interpretation of the effect of thymine concentration on replication velocity is correct there should be a corresponding effect of thymine concentration on the intracellular concentration of thymidine triphosphate (dTTP). This is found (see figure 7*a*). Several features of the data shown are important in the context of this Review. First, it can be seen that the dTTP concentration corresponding to a given thymine concentration in 15T$^-$ is higher than that in CR 34 in accordance with their differing thymine requirements. Secondly (although the data are not shown in the figure), the plateau concentrations of dTTP in both strains is lower than that found in *thy*$^+$ derivatives of them. In 15T$^-$ the maximum attainable dTTP concentration is about half and in CR 34 it is about one-fifth of that found in the corresponding *thy*$^+$ strains. We had initially assumed (Beacham, Barth & Pritchard 1968) that this deficit might be due to the fact that the supply of P-1-dRib in such strains places a limit on the internal dTTP concentration attainable. Subsequent experiments have shown that this explanation is incorrect. If a deoxynucleoside is added to the growth medium to increase the internal concentration of P-1-dRib it is found (figure 7*b*) that no increase in the maximum dTTP concentration is achieved (Beacham *et al.* 1971).

The reason for low maximum dTTP concentration in *thy*$^-$ strains is clarified to some extent by adding thymidine to the growth medium instead of thymine (thus bypassing the need for an internal source of P-1-dRib). It can be seen (figure 7*b*) that the dTTP concentration approaches the wild-type level at very low thymidine concentrations and then *falls* as the thymidine concentration is increased to the plateau level attained with thymine. It therefore seems likely that thymidine itself inhibits one of the later steps in its own conversion to dTTP and that this is the reason for the failure to achieve wild-type concentrations irrespective of the quantity of thymine or thymidine added to the growth medium. The inhibitory effect of thymidine on the dTTP concentration is markedly less in the case of 15T$^-$ than in CR 34 (Beacham *et al.* 1971). We have suggested that a mutation causing this difference underlies the 'super-low' property of 15T$^-$.

(*c*) *Labelling techniques*

Isotopically labelled thymine and thymidine are routinely used in experiments in which it is necessary to label DNA or to determine the kinetics of DNA synthesis. It will be appreciated that the use of labelled thymine alone will often be unsuitable since if low concentrations are used to conserve label the culture will have an atypical replication velocity, except perhaps in 'super-low' strains. (In the case of the super-low strain 15T$^-$ there is some evidence (Zaritsky & Pritchard 1971) that even with thymine concentrations of 10 μg/ml in the growth medium the replication velocity is not maximal.) Particularly serious errors will be introduced in kinetic studies on DNA synthesis if the concentration of thymine is changed during the course of an experiment. Labelled thymidine does not suffer from this disadvantage, but its rapid degradation to thymine by thymidine phosphorylase limits its usefulness to pulse-labelling experiments unless thymidine phosphorylase negative (*tpp*$^-$) strains are employed.†

A generally useful technique for long-term labelling of both *thy*$^-$ and *thy*$^+$ strains is to use a

† Thymidine is also degraded slowly by uridine phosphorylase, so some loss will occur even in *tpp*$^-$ strains (Beacham & Pritchard 1971). It is important to note in this connexion that 5-bromodeoxyuridine, a thymine analogue which is often used as a density label for DNA, is accepted as a substrate in this enzyme much more readily than thymidine (Beacham & Pritchard 1971). This accounts for the hitherto puzzling finding that a *tpp*$^-$ strain degraded the analogue at about half the rate found in *tpp*$^+$ strains (Fangman 1969).

mixture of thymine at low concentrations (1 μg/ml is sufficient) and deoxyguanosine at high concentrations (200 μg/ml or more) as a source of P-1-dRib. With this technique care must be taken that sufficient deoxyguanosine is added to the growth medium to ensure that it is not completely degraded to guanine and P-1-dRib by purine nucleoside phosphorylase during the course of the experiment. Another method is to use a low concentration of labelled thymidine in the presence of a high concentration uridine (Spratt & Rowbury 1970) making use of the observation that thymidine phosphorylase is inhibited by uridine (Budman & Pardee 1967; Beacham, Eisenstark, Barth & Pritchard 1968).

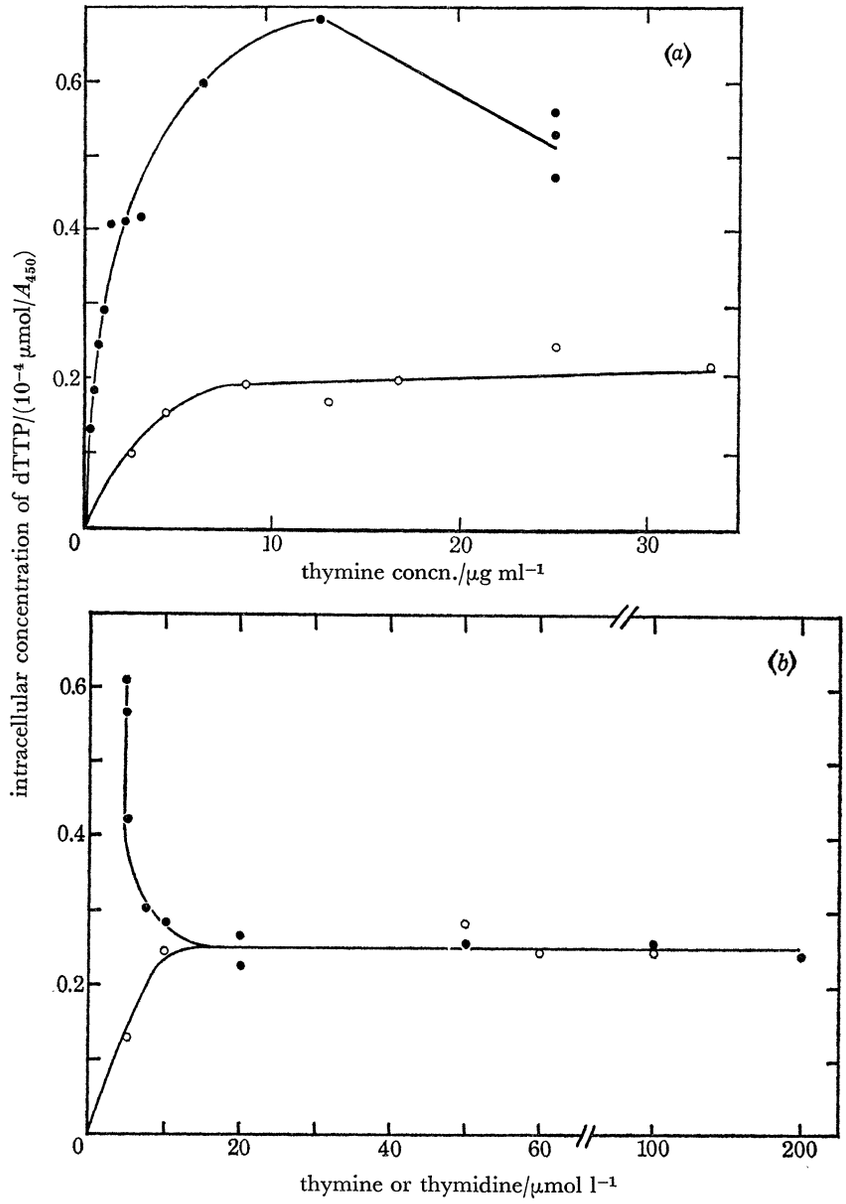

FIGURE 7. (*a*) Effect of thymine concentration on the intracellular concentration of thymidine triphosphate in *E. coli* 15T⁻ (●) and *E. coli* CR 34 (○). (*b*) Effect of thymidine concentration (●) or thymine concentration in the presence of deoxyguanosine (1 mM) (○) on the intracellular concentration of thymidine triphosphate in CR 34. Data in (*a*) and (*b*) taken from Beacham *et al.* (1971). Intracellular concentration of thymidine triphosphate (dTTP) is expressed here in μmol/A_{450}, where A_{450} is unit absorbance proportional to the dry mass of bacterial cells.

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A most important point which emerges in connexion with labelling of *thy*$^+$ strains either with thymidine alone or with a mixture of thymine and deoxyguanosine is that the inhibitory effect of thymidine on dTTP synthesis leads to the paradoxical situation that the *higher* the concentration of thymidine (or thymine in a thymine-deoxyguanosine mixture) the *smaller* the contribution of the externally supplied compound to the dTTP pool and the smaller the total pool. Thus for maximum incorporation of label the lowest possible concentration of thymidine or thymine should be used.

An additional problem in experiments in which a deoxynucleoside such as thymidine or deoxyguanosine is added to the growth medium when using Tlr. strains is that the growth of those which are deoxyriboaldolase negative (*dra*$^-$) is inhibited as a result of a toxic accumulation of dRib-5-P. This problem does not arise with phosphopentomutase negative Tlr. strains.

Clearly great care must be taken in the culture and labelling of both *thy*$^+$ and *thy*$^-$ strains in order to obtain quantitatively meaningful data. Extrapolations from the behaviour of one strain to another are dangerous.

Shift and velocity-jump experiments

Kjeldgaard *et al.* (1958) extended their initial analysis of cell size and composition as a function of growth rate by examining the kinetics of a transition from one steady-state growth rate to another following an abrupt enrichment of the growth medium. An enrichment of this sort was termed a 'shift-up' and this kind of experiment has proved particularly useful in elucidating the factors determining cell composition, particularly with respect to protein and RNA and the factors controlling the rates of synthesis of these macromolecules (see Maaløe & Kjeldgaard 1966; Koch 1971). The usefulness of experiments of this type is limited by the substantial adjustments in cell metabolism which are involved in such a transition. Indeed, the reciprocal transition (a 'shift-down') cannot be usefully exploited because in this case growth of the culture normally stops for a prolonged period.

Experiments of the type shown in figure 4 are analogous to shift experiments. Instead of changing the growth rate while maintaining a constant replication time, we introduce an abrupt change in the replication time of the chromosome while maintaining a constant growth rate. We have described such experiments as step-up or step-down transitions (Zaritsky & Pritchard 1971) and colloquially as velocity-jump experiments. The advantage of velocity-jump experiments is that changes in DNA concentration and relative gene dosage can be induced without complicating changes in cell metabolism. They are particularly useful analytical tools for investigating the factors controlling cell size and shape, as will be discussed in later sections of this Review. We have also used them to investigate the effect of gene concentration on the rate of protein synthesis in *E. coli* and to determine the relation between chromosome and episome replication.

(*a*) *Gene output as a function of gene concentration*

If the initial rate of synthesis of β-galactosidase following addition of the inducer IPTG (isopropyl thio β-galactoside) is measured at successive times in synchronous cultures of *E. coli* the pattern shown in figure 8*a* is found (Helmstetter 1968; Zeuthen & Pato 1971): the rate per cell is constant throughout the entire cell cycle apart from a discrete doubling in rate which occurs at the time the number of copies of the gene specifying this enzyme doubles as a result of replication. A similar pattern is observed for other inducible enzymes (Helmstetter 1968), the

doubling in rate occurring at a unique cell age which is different for different genes according to their map location. An informative way of presenting results of experiments of the type shown in figure 8*a* is to plot the differential rate of enzyme synthesis rather than the rate per cell as a function of cell age. This is done in figure 8*b* on the assumption that the rate of total protein synthesis increases exponentially (Ecker & Kokaisl 1969) during the cell cycle.

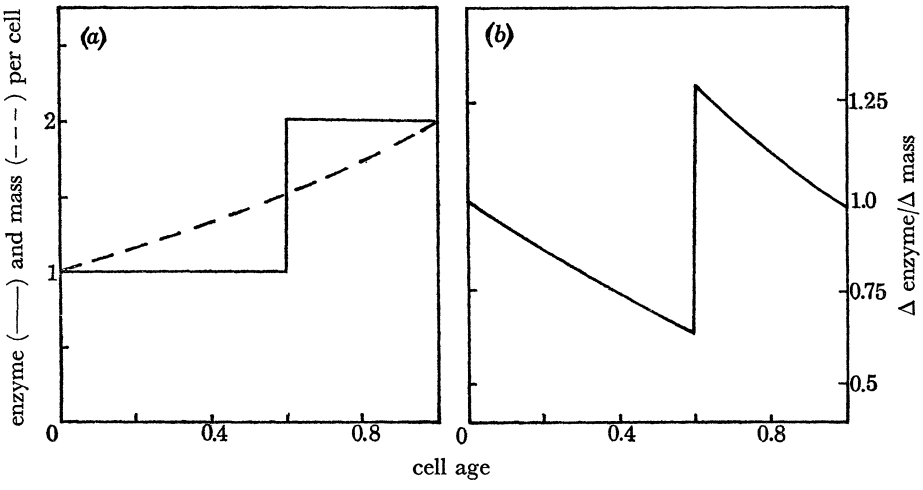

FIGURE 8. Diagrammatic and idealized representation of capacity to synthesize β-galactosidase as a function of cell age. (*a*) On a per cell basis; (*b*) the differential rate. The dashed line in (*a*) represents total mass (or protein) per cell.

When the data are plotted in this way one sees that the differential rate of synthesis of β-galactosidase *per gene* falls continuously. In other words, although the overall capacity of the cell to synthesize protein is increasing continuously, that of a fully derepressed gene remains constant with the result that the differential rate of synthesis of its product decreases. So far as I know this interesting relationship has not been discussed hitherto. One interpretation might be that the overall capacity to synthesise protein increases during the cell cycle because the concentration (or quantity) of a common element involved in transcription or translation is increasing, but there is competition for this element so that the progressive increase in the amount of DNA (the total number of genes) during the cell cycle results in a decreasing proportion of it being available to any fully derepressed gene. A second explanation is that the overall capacity to synthesize protein increases as a direct result of the increasing amount of DNA per cell, the implication being that the rate limiting factor in overall protein synthesis is the amount of DNA. In other words, the continuous increase in the rate of protein synthesis represents a summation of the step-wise doubling in output from each gene as it is replicated. These are both competition hypotheses at the level of DNA and imply either that the majority of genes are not subject to regulation or that their degree of repression does not change during the cell cycle. They both predict that the differential rate of synthesis of a fully derepressed gene should be a function of its *relative dosage*. A third, and more realistic, hypothesis is that the activity of the majority of genes is controlled by end products and that they become progressively more derepressed during the cell cycle because (*a*) the rate of withdrawal of these end products increases, (*b*) the concentration of enzymes involved in their synthesis would fall if their degree of repression was constant and (*c*) cell volume is increasing. The constant rate of protein synthesis by a fully derepressed gene (figure 8) implies on this hypothesis that the increasing demand for any

common rate-limiting element is matched by an increase in the amount available such that its concentration remains constant. This hypothesis predicts that the differential rate of synthesis by a gene in a fully derepressed state will be proportional to the *gene concentration* (gene:mass ratio) and independent of its relative dosage.

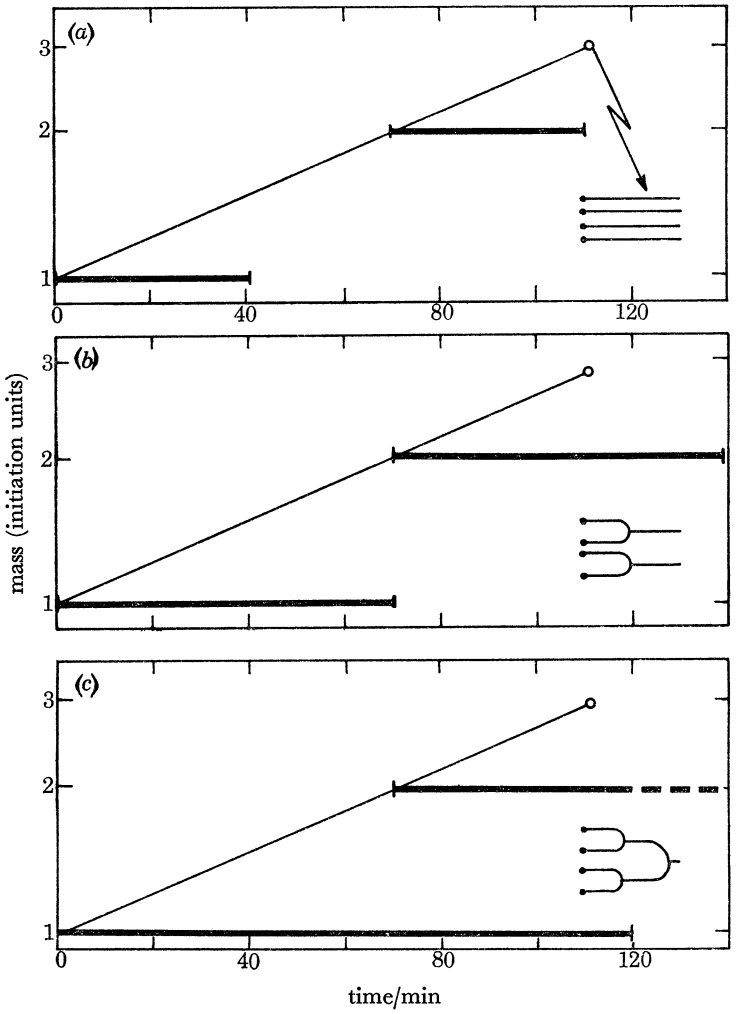

FIGURE 9. Effect of different replication velocities on DNA concentration and on the configuration of an average chromosome. Details as for figure 3.

The velocity jump technique provides us with a method for investigating the relative importance of gene concentration and relative gene dosage in determining the output of a fully derepressed (or fully repressed) gene. I have pointed out that the change in average gene concentration which results from a change in growth rate in unsynchronized exponential cultures will be different for different genes depending on their location relative to the chromosome origin and terminus. Similarly, the magnitude of the change in gene concentration resulting from a change in the replication time of the chromosome at one growth rate should also depend in the same way on the location of the gene in question as is shown diagrammatically in figure 9.

We can compute the expected change in average gene concentration (Δgc) for a given change

in C (ΔC) according to the fractional distance (x) of the gene in question from the chromosome origin. The relation is found to be (M. G. Chandler, personal communication)

$$\Delta gc = 2^{\Delta C x/\tau}. \quad (4)$$

In deriving this expression we have assumed that the average concentration of chromosome origins is constant and independent of C (i.e. the initiation mass is constant).

It is important to note that the change in gene concentration is not affected by the absolute values of C (which may be difficult to ascertain accurately) but only on the difference in C due to the change in thymine concentration used.

If we now compare the change in gene concentration for a given ΔC of two genes a and b with different chromosome locations x_a and x_b we can show (M. G. Chandler, personal communication) that

$$\lg (\Delta gc)_a / \lg (\Delta gc)_b = x_a/x_b. \quad (5)$$

This means that if the differential rate of enzyme synthesis of a fully derepressed or fully repressed gene is proportional to its concentration then by comparing the size of the change in differential rate for a given change in C we can find a value for x_a/x_b without needing to know ΔC. Then by repeating the operation for a series of pairs of genes whose map locations are known we can find whether there is a unique solution for the location of the chromosome origin. If we find a unique location for the origin by this technique and it is in agreement with the location determined by independent methods we can conclude that the data are consistent with the hypothesis in that gene output is proportional to gene concentration and that initiation occurs at a constant mass to chromosome origin ratio.

The kind of result we obtain in experiments of this kind is shown in figure 10. Notice that in a velocity-jump experiment the differential rate of β-galactosidase synthesis increases as expected since the gene coding for this enzyme is located some distance from the origin (an identical change in the differential rate of β-galactosidase is observed in the case of basal synthesis in the absence of inducer). Notice also there is a negligible increase in the differential rate of synthesis of tryptophanase which on the same basis must be located close to the origin. We have examined the change in gene output from three other genes in this way (thymidine phosphorylase, D-serine deaminase and tryptophan synthetase) and find (M. G. Chandler & R. H. Pritchard, unpublished data) from a pairwise comparison of the data according to equation (5) that there is a remarkable consistency in the computed location of the origin somewhere between map positions 63.5 and 70.5, the average value being 67 min on the *E. coli* map. We also find that a unique location is only obtained if we assume that replication is bi-directional. The position of the origin determined by this method is close to that found using totally independent methods by Masters & Broda (1971) and Bird *et al.* (1972).

Having located the chromosome origin in this way and therefore having solved for x for any gene we can compute ΔC from the change in the differential rate of synthesis for the gene (equation (4)) and compare this value with ΔC computed from the change in DNA to mass ratio (figure 10, top curve) using equation (2). Once again we get a very close agreement between the two methods.

We can now go through the whole operation again, but this time assuming that gene output is proportional to relative gene dosage. The mathematical treatment need not be given here. Suffice to say that by means of this assumption we cannot obtain a unique location for the chromosome origin. Moreover, if we assume a location for the chromosome origin and calculate ΔC from the

change in the differential rate of synthesis we get a different result for each enzyme and no agreement with ΔC calculated from the change in DNA to mass ratio.

We are therefore able to conclude that the changes in gene output and in DNA concentration in velocity-jump experiments are qualitatively and quantitatively consistent only with the following postulates:

(1) The initiation mass is constant and independent of the thymine concentration in the growth medium at one growth rate.

(2) Rounds are initiated at a unique site, the best estimate for the location of this site being at 67 min on the *E. coli* map. Replication is bidirectional.

(3) The differential rate of protein synthesis by a gene in a fully induced or repressed rate is proportional to the gene concentration and independent of the relative gene dosage.

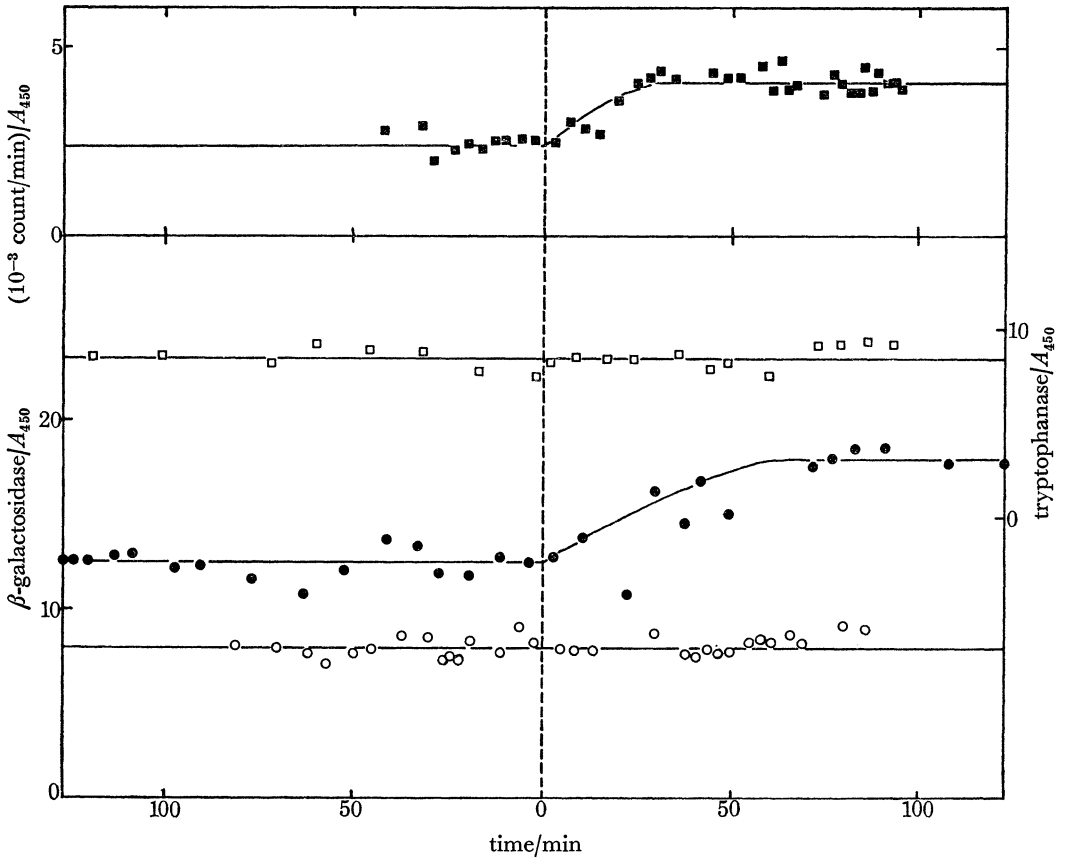

FIGURE 10. Effect of a step-up in thymine concentration on (■) DNA concentration; (□) the induced rate of synthesis of tryptophanase; (●) the induced rate of synthesis of β-galactosidase; (○) the induced rate of synthesis of β-galactosidase in an F-*lac*$^+$ strain carrying a chomosome deletion of the *lac* gene (unpublished data of M. Chandler). Cultures contained 1 μg/ml thymine before time zero and 20 μg/ml after this time. The medium was a glycerol synthetic medium supplemented with casamino acids. DNA concentration was measured in cultures uniformly labelled with [^{14}C]thymine. Tryptophanase and β-galactosidase activities were measured after a brief exposure to inducer.

The conclusion that the output of fully induced or fully repressed genes is not affected by changes in their relative dosage means that such changes do not alter the availability (or concentration) of any rate-limiting element involved in transcription or translation. This, in turn, implies that there can be very few genes which make significant demands on the cell's

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biosynthetic resources, the output from which is not compensated (by regulation) in response to changes in their concentration. This conclusion, it should be emphasized, is not incompatible with the hypothesis (Maaløe 1969) that genes specifying ribosomal proteins are not subject to regulation since provided these are the only major genes of this type the fraction of any common rate-limiting element employed in their transcription or translation will be inversely related to the fraction similarly employed by all other (regulated) genes. More difficult to equate with Maaløe's hypothesis is the fact that while the rate of synthesis of rRNA increases continuously during each cell cycle (Dennis 1971) the capacity to synthesize β-galactosidase (and presumably its messenger) per gene remains constant. If both genes shared a common rate-limiting element their relative rates of synthesis should always be identical.

(*b*) *The concentration of a plasmid as a function of C*

Many bacterial species can harbour extra chromosomal particles of DNA. In the case of certain of these episomes (such as the sex-factor F) we know that the number of particles per cell is small yet the association is extremely stable. It follows that the frequency of replication of such particles and their segregation during cell division must be well regulated. It has been generally assumed (following Jacob & Wollman 1961) that control of F replication is independent of that of the host chromosome. It has also been generally assumed that the mechanism of control of F replication and chromosome replication are similar (Jacob, Brenner & Cuzin 1963; Pritchard *et al.* 1969), but neither of these assumptions have been verified unequivocally. An obvious alternative would be that F replication is coupled in some way to chromosome replication and is not, in this sense, autonomous.

Our finding, described in the previous section of this Review, that the differential rate of synthesis of genes in an unregulated state is proportional to gene concentration provides a simple method by which it can be determined whether F replication is coupled to any event in the replication cycle of the host chromosome other than to initiation. We infected a *thy*$^-$ strain (with the *lac* gene deleted) with an F prime particle carrying the *lac* gene. Using the procedure described previously we measured the differential rate of synthesis of β-galactosidase before and after a transition from one thymine concentration to a higher one. The results of such an experiment are shown in figure 10 (bottom curve). It can be seen that in contrast to the results obtained when the *lac* gene occupies its normal chromosomal location there is no detectable change in the differential rate of synthesis of the enzyme when it is carried by the F particle. The implication of this experiment is that the concentration of the F particle (the F to mass ratio) is not affected by a change in replication velocity. Either replication of the particle must be coupled to initiation of rounds of chromosome replication (i.e. it behaves like a gene located near the chromosome origin) or, if it is autonomous, its replication must occur at a constant mass to particle ratio which may be the same or different from that at which initiation of chromosome replication takes place.

Cell size as a function of growth rate

An important advance in the development of our understanding of the relation between cell size and growth rate was the demonstration by Cooper & Helmstetter (1968) that over a range of doubling times between about 70 and 20 min cell division follows at a constant time (approximately 25 min in batch cultures) after termination of rounds of chromosome replication in

E. coli B/r. They designated the time interval D and suggested (Helmstetter *et al.* 1968) that termination of rounds provides a signal which initiates cell division, cell separation occurring 25 min later. With this additional information relating chromosome replication to cell division we can see diagrammatically (figure 11) why cell size increases with increasing growth rate provided once again it is assumed that initiation of rounds of chromosome replication occurs

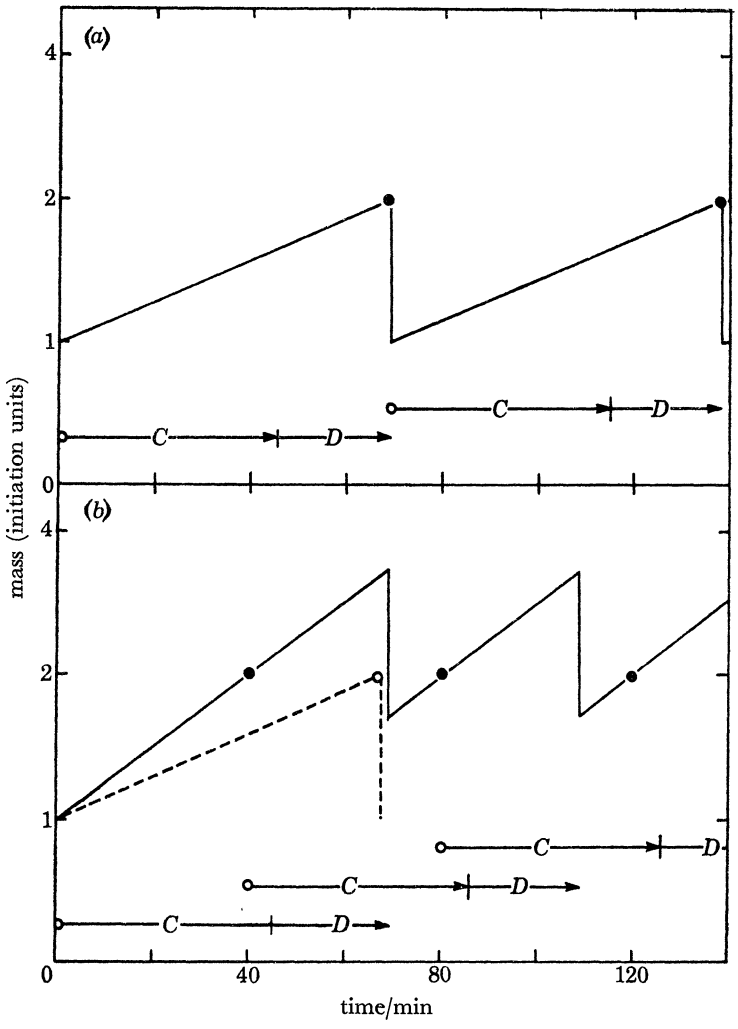

FIGURE 11. Effect of increasing growth rate on cell size. (*a*) Growth and division of a single cell with a size of one initiation mass after division is shown. The time of initiation of a new round of replication is shown thus ●. (*b*) The effect of an increase in growth rate on cell size and the time of initiation of a new round of replication. C is assumed to be 45 min and in (*b*) rounds of replication overlap since the doubling time of the culture mass is 40 min. The dashed curve in (*b*) reproduces the growth curve in (*a*) for comparison.

at a constant mass to chromosome origin ratio. In addition, we can predict that the relation between average cell mass and growth rate in a steady state of exponential growth will be given by the expression (Pritchard *et al.* 1969):

$$\bar{M} = k \times 2^{(C+D)/\tau}, \tag{6}$$

where k is the constant used previously. The slope of the curve relating average cell mass with growth rate in *S. typhimurium* (figure 1) is that expected from the above relation if in this strain, $C+D$ has a value of 63 min, and Spratt & Rowbury (1971) have provided additional evidence

that C and D have values in *S. typhimurium* which are similar to those found in *E. coli* B/r. The constancy and length of D may therefore have wide applicability.

The constant time between termination of a round of replication and cell separation is not at present understood. The notion that termination is normally a precondition for cell division is supported by a variety of observations. Thus when DNA synthesis is blocked by thymine starvation, by nalidixic acid (a specific inhibitor of DNA synthesis) or ultraviolet irradiation in a steady-state exponential culture division continues for about 20 min and the fraction of the population which divides is approximately that which can be computed to be in the D period

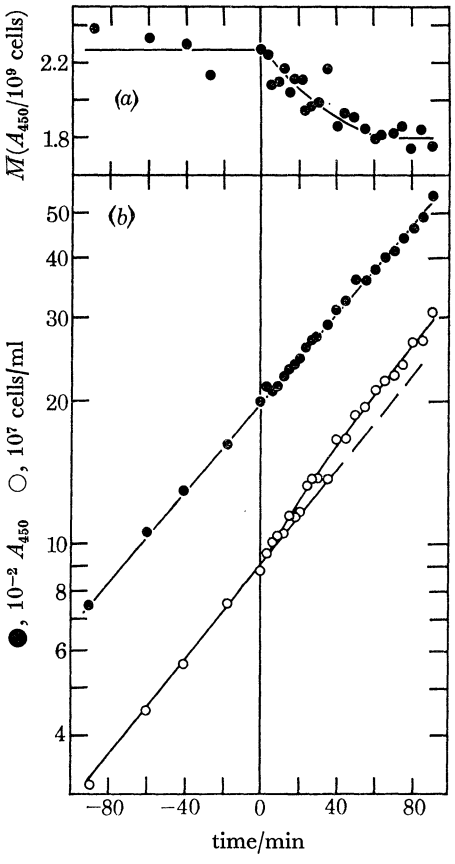

FIGURE 12. Effect of an increase in thymine concentration on the rate of cell division and average cell size. To steady-state exponential culture of *E. coli* 15T⁻ in glycerol synthetic medium containing 0.5 μg/ml thymine additional thymine was added at time zero to give a concentration of 5.0 μg/ml: (*b*) shows absorbance and particle number as a function of time. (*a*) shows average cell size ($\bar{M}$) computed from the data in (*b*). (Reproduced from Zaritsky & Pritchard (1973).)

(Helmstetter & Pierucci 1968). In addition, these authors, and Clark (1968), have shown that the cells which divide under these conditions are the 'old' cells which have completed a round of replication. In thermosensitive mutants of *E. coli* and *S. typhimurium*, in which initiation of new rounds of replication is blocked at the non-permissive temperature although rounds in progress are completed (Hirota, Mordoh & Jacob 1970; Spratt & Rowbury 1970), normal cell division continues for a longer period (about 70, i.e. $C+D$ min) and the increment in cell number during this time is much greater, as would be expected since in addition to those cells which were in the D period at the time of the shift to the non-permissive temperature

termination of a round of replication will occur in the majority of cells (Spratt & Rowbury 1971). Although the division behaviour of cultures in these two kinds of experiment is consistent with the notion that termination is a precondition for cell division the agreement between the observed time course and extent of residual division and expectation could be fortuitous: inhibition of DNA synthesis and incubation of thermosensitive mutants at the non-permissive temperature are both lethal treatments which ultimately stop growth and division in all cells.

The velocity jump technique provides a method for investigating the relation between termination and division which avoids this objection since one can introduce a transient change in the frequency of arrival of replication forks at the terminus without disturbing the growth rate. In addition, since *C* can be specifically modified it is possible to investigate the temporal relation between termination and cell division with this technique.

If a steady-state exponential culture growing on a low concentration of thymine is stepped-up to a higher concentration we will expect the rate of arrival of replication forks at the terminus to increase transiently. If there is a relation between termination and cell division we will consequently expect to see a corresponding transient acceleration in the rate of cell division and reduction in average cell mass. As can be seen from figure 12, which shows the results of such an experiment, this prediction is upheld.

This kind of experiment illustrates most clearly how the velocity jump technique can be used to ask what events in the cell cycle are coupled directly or indirectly to chromosome replication. The concommitant change in the rate of DNA synthesis and the rate of cell division shows that the two are coupled in some way. This result should be compared with the experiment shown in figure 10 in which we show that the rate of replication of an F particle is not affected by changing *C* indicating an absence of coupling, other than to initiation, between chromosome replication and F replication.

A notable feature of the experiment shown in figure 12 is that average cell size, and therefore the rate of cell division, begins to change with no detectable delay after a step-up in thymine concentration. If termination provided a signal for division which always occurred 25 min later, we would expect there to be a delay of 25 min after the transition before the rate of cell division accelerated. The absence of such a delay must mean either that in the strains used or because of the thymine limitation imposed, *D* is much shorter than it is in *E. coli* B/r, or that there is a more direct and unexplained relation between replication velocity and cell division.

An extensive analysis of the effect of changing *C* on cell division in a *thy*$^-$ derivative of *E. coli* B/r has been made by Mr P. Meacock in my laboratory. In step-up experiments with this strain he obtains results like those shown in figure 12. In step-down experiments, on the other hand, there is a delay of about 20 min before the rate of cell division begins to respond to the change in *C*. This difference suggests that in cultures growing on low concentrations of thymine the longer *C* period is associated with a shorter *D* period. In other words, *C* increases at the expense of *D*. That this is so, he has confirmed by measuring the length of *D* more directly in synchronous cultures of this strain growing on different thymine concentrations using the technique of Cooper & Helmstetter (1968). An important feature of his data is that the reduction in the length of *D* is always less than the corresponding increase in *C*. We must conclude that although termination is normally a precondition for cell division other factors influence the timing of division once termination has occurred.

For termination to make any sense as a division signal it could be argued that each act of termination should permit only one act of division, and the experiments in which it has been

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shown that blocking DNA synthesis by nalidixic acid prevents further division by the majority of cells in a culture are in effect a test of this premise. Paradoxically however, in many thermosensitive initiation defective stains the initial phase of division following a shift to the non-permissive temperature is followed after a lag by a resumption of division leading to the production of cells containing no chromosomal DNA (Hirota *et al.* 1968; Spratt & Rowbury 1971). Continued division and production of DNA-less cells also occurs in some thermosensitive strains in which DNA synthesis is blocked immediately at the non-permissive temperatures (see Hirota & Ricard 1972; Inouye 1969). In some cases evidence has been obtained that the production of DNA-less cells depends on the presence of additional mutations in the thermosensitive strains (see Hirota & Ricard 1972; Inouye 1971), but in other cases this does not appear to be so (Spratt & Rowbury 1971). These results are not compatible with a 1:1 relation between termination of rounds and cell division.

Before considering how these paradoxical observations on the role of termination in cell division and the temporal relation between the two might be resolved I shall need to discuss another revealing effect of variation in growth rate and replication velocity on bacterial cells.

Cell shape

Cells of *E. coli* are cylinders with rounded ends. They increase in size under steady-state exponential conditions by extending only in length – cells at birth have the same diameter as cells about to divide (Marr, Harvey & Trentini 1966). The fact that they extend only in length is presumably a reflexion of the mode of synthesis of the cell envelope (called here the cell wall). It might therefore be anticipated that the increase in average cell size which accompanies an increase in growth rate would be reflected in an increase only in cell length. This is not so (see Schaechter *et al.* 1958). An increase in growth rate results in an increase in both length and girth. Since at any one growth rate cells extend only in length the change in girth must be a once and for all event which occurs during the transition from one growth rate to another.

There seems to be only one reasonable explanation for this effect. This is that when growth rate increases the rate of wall synthesis is not increased proportionately so that there is a reduction in the wall to mass ratio, which must lead to a decrease in the surface to volume ratio if the cell is to maintain a constant density. One way in which a decrease in the differential rate of wall synthesis with increasing growth rate might be brought about would be for the rate of wall synthesis to be determined by the rate of synthesis of a precursor which is a protein specified by an unregulated gene. The rate of synthesis of this precursor would be proportional to the number of gene copies per cell (see, for example β-galactosidase (figure 8)) and if this gene were located near to the chromosome terminus the differential rate of synthesis of its product would fall with increasing growth rate for the reason I have discussed earlier. This suggestion could only be entertained seriously if it were shown that the rate of wall synthesis (and cell volume increase) is linear with a discrete doubling in rate occurring at the time the gene is replicated. There have been many attempts to determine whether cells of *E. coli* increase linearly or exponentially in volume (see Kubitschek 1970, 1971; Ward & Glaser 1971), but over the twofold range of volumes which occurs during the one cell cycle the difference between a linear and an exponential mode is so small as to make it difficult to distinguish between them. The weight of evidence nevertheless seems to fit more closely to a linear rather than an exponential mode of volume increase. In addition, Hoffman, Messer & Schwarz (1972) have measured the rate of incorporation of D-

glutamic acid into the mucopeptide component of the cell wall in synchronous cultures of *E. coli* B/r and obtained data suggesting that the rate is linear with a doubling in rate occurring in the middle of the cell cycle. It is not possible to relate the timing of this doubling in rate to a particular stage in the replication cycle since these authors used a *thy*$^-$ strain and did not determine *C* under the conditions used.

The notion of a linear rate of wall synthesis determined by the output of an unregulated distally located gene leads to a particularly striking prediction concerning the relation between replication velocity and cell shape. According to equation (6) an increase in *C* will lead to an increase in average cell size and an effect of *C* on cell size has been found experimentally (figure 12). Now if we lengthen *C* the concentration of a distal gene will decrease (figure 9), so that although the cells will be larger the wall to mass ratio will fall and the cell will of necessity become shorter and fatter. We have made some preliminary determinations of the effect of changing *C* on cell shape (Zaritsky & Pritchard 1973) with results that are summarized in table 1. From rows 1 and 4 it will be seen that in keeping with the initial observations of Schaechter *et al.* (1958) we observe that the increase in cell size associated with an increase in growth rate is associated with an increase in both length and width. When size is increased by increasing *C*, however, the cells increase only in width and appear to become shorter (rows 1–3). They nevertheless remain typical rods. It is difficult to conceive of any explanation for this effect other than that the longer *C* the less wall material that can be synthesized in one cell generation.

TABLE 1. DIMENSIONS OF *E. COLI* 15 UNDER DIFFERENT CONDITIONS

genotype	carbon source	thymine concentration μg/ml	relative volume	relative radius	relative length (calculated)
thy$^+$	glycerol (τ = 60′)	—	1.00	1.00	1.00
thy$^-$	glycerol	2.00	1.07	1.17	0.78
thy$^-$	glycerol	0.30	1.56	1.41	0.78
thy$^+$	glucose (τ = 40′)	—	1.60	1.16	1.23

Average cell volumes of *E. coli* 15T$^-$ and a *thy*$^+$ derivative of it were obtained from the mean of the pulse height distribution obtained with the aid of a modified Coulter electronic particle counter using steady-state exponential cultures. Radii were measured on electron micrographs of fixed preparations of the same cultures and average lengths were calculated from the volume and radius measurements assuming the cells to be cylinders. (Data from Zaritsky & Pritchard (1973).)

The fact that we observe a reduction in the surface to volume ratio in experiments in which there is no significant change in the composition of the growth medium and no change in growth rate also makes unlikely another possible interpretation of the similar effect which occurs when the growth rate is increased. This is that the reduced differential rate of wall synthesis is due to increase of specific or non-specific (e.g. catabolite) repression of a gene specifying a key protein involved in wall synthesis.

If the reduction in the wall to mass ratio resulting from an increase in *C* or an increase in growth rate is due to a reduction in the concentration of a wall precursor gene, and if we make the reasonable assumption that the rate of transcription of this gene is proportional to the growth rate, then we can calculate the expected relation between average surface area per cell and growth rate as a function of the distance of this gene from the chromosome origin. Existing

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experimental data from *S. typhimurium* are in closest agreement with a location for the postulated gene about two-thirds of the distance between the chromosome origin and terminus (see figure 13).†

Although I have suggested that the linear rate of volume increase is due to a linear rate of synthesis of a rate-limiting precursor an alternative possibility is that volume increases linearly because addition of new cell material takes place at one or a limited number of zones encircling the cell and the rate of extension is limited by the number of available sites for addition of new material in these zones rather than by the amount of precursor available (Zaritsky & Pritchard 1973). A doubling in rate of synthesis would require a doubling in the number of such growth zones and, for the reasons already discussed, the formation of new growth zones would need to be

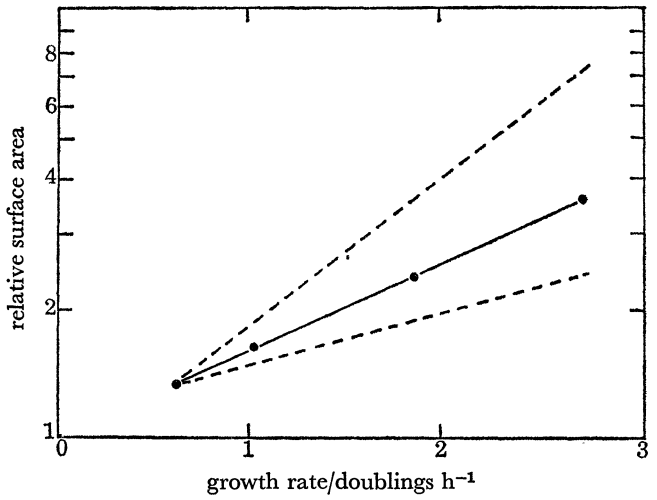

FIGURE 13. Average surface area of *S. typhimurium* as a function of growth rate. The values are calculated from average mass per cell and radius per cell using the data given in Schaechter *et al.* (1958), assuming the cells to be cylinders with hemispherical ends. The data are tested against the model that average surface area is proportional to growth rate using the following relation (M. G. Chandler, personal communication):

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

where $\bar{F}$ is average gene concentration, x is the fractional distance of the gene from the chromosome origin and the other terms are as defined previously. Using $C = 45$ min and $D = 25$ min, regression analysis gives a value for x of 0.64. The upper dashed line shows the expected change in surface area if the gene were located at the chromosome origin ($x = 0$) and the lower dashed line the expected change if the gene were located at the terminus ($x = 1$).

associated in some way with a late event in the replication cycle. The mode of incorporation of new material into various components of the cell wall in Gram-negative bacteria has been the subject of much controversy but there seems to be increasing evidence that synthesis of the mucopeptide layer occurs primarily in a single centrally located zone in *E. coli* (Schwarz, Asmus & Frank 1969; Ryter, Hirota & Schwarz 1973). Zonal growth of the inner cytoplasmic membrane is also indicated by the data obtained by Kepes & Autissier (1972).

It should be noted that zonal growth is not incompatible with a precursor limitation model for

† The assumption that the output of an unregulated gene will be proportional to the growth rate μ can be questioned. Arguments have been presented (Maaløe 1969) which suggest that the output of such a gene might be more nearly proportional to μ^2. In this case the increase in surface area with growth rate would be close to that expected if the gene concerned were located at the terminus.

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linear increase in volume, although the absence of zonal growth would perhaps make a site-limitation model less plausible. The two models are very similar, but should be distinguishable experimentally because they lead to quantitatively different predictions about the way average cell length, cell width and surface area will change with changes in growth rate and replication velocity.

The implication of a linear rate of wall synthesis and an exponential rate of increase in cell mass is that mass and volume are never increasing at the same rate. During part of the cell cycle new mass will be accumulating faster than volume is increasing to accommodate it. During the remainder of the cycle the inverse will be the case (figures 14, 15*a*). If we suppose further that a growing cell seeks to maintain constant relative concentrations of intracellular components,

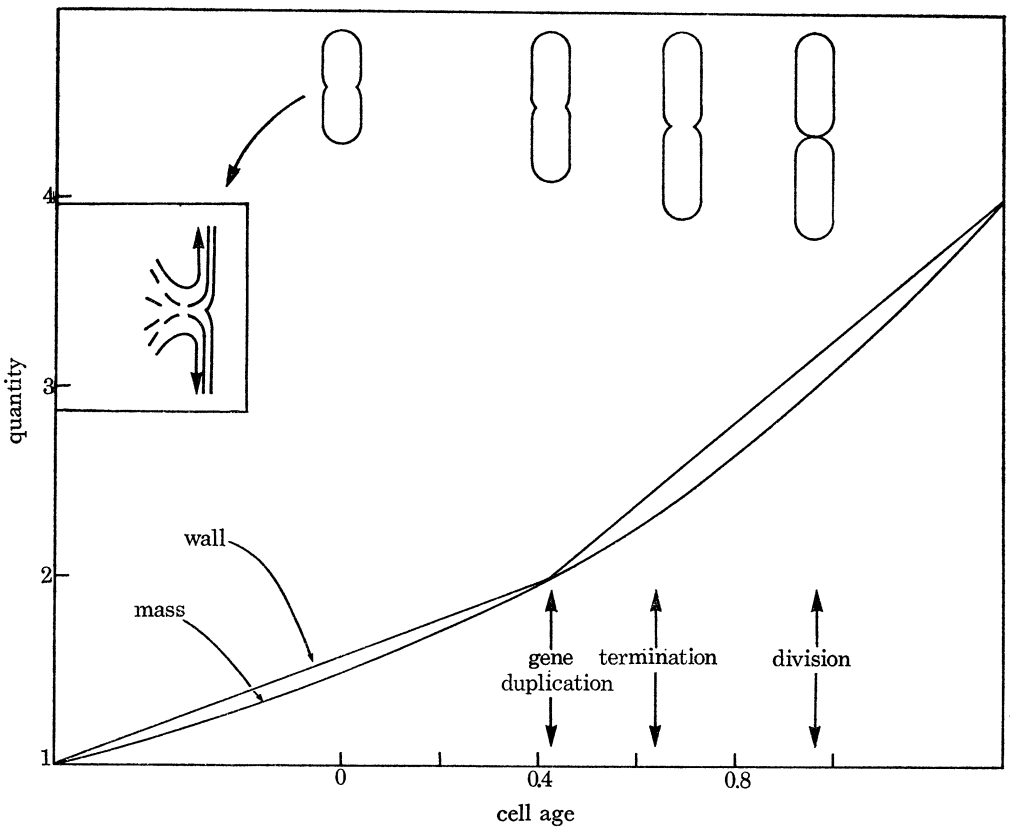

FIGURE 14. Growth and division in *E. coli*. The curves show how a linear rate of wall synthesis and exponential rate of mass increase leads to a relative rate of synthesis of the two components which is continuously changing. The cell ages at which duplication of the rate limiting precursor gene, termination and cell division occur are shown for a cell with a doubling time of 70 min. The cells and the appearance of the nascent septum are drawn to illustrate the model not to indicate their actual morphology.

including the low molecular mass components which will contribute significantly to the osmotic pressure of the cytoplasm, there will be a corresponding oscillation in the internal hydrostatic pressure. This pattern of growth provides a possible basis for understanding how, as opposed to why, cells which extend only in length under steady state conditions have different diameters at different growth rates and replication velocities. It may also form the basis for understanding: (i) the timing of the transition between lengthwise addition of new wall and centripetal addition of new wall to form a septum which occurs once in every cell cycle, (ii) the inhibition of cell

division when termination is prevented, and (iii) the continued division and production of DNA-less cells in initiation defective strains.

A model for cell division

Higgins & Shockman (1970, 1971) have provided convincing morphological evidence that in the spherical gram-positive bacterium *Streptococcus faecalis* new wall is laid down primarily at the leading edge of a nascent cross-wall, the peripheral wall being formed by the peeling apart of this nascent septum into two halves which migrate outward as the cell increases in volume. Let us suppose that new wall subunits are added to existing wall in a similar fashion in *E. coli* (see inset figure 14) even though morphological evidence for this is lacking in this species. In other words we suppose that new wall is added as if the cell were always attempting to form a septum. A growing cell is increasing in mass, however. In seeking to maintain a constant relative concentration of intracellular components it will be taking up water and the internal hydrostatic pressure tending to increase. The increasing pressure is relieved by extension of the cell in length, this being possible because the newly synthesized wall material can be drawn out along the periphery of the cell. Even though all new wall units added to the leading edge of the nascent cross-wall are used for length extension internal hydrostatic pressure will increase during the early part of the cell cycle. When the linear rate of wall synthesis doubles however, the hydrostatic pressure will rapidly fall as the rate of length extension increases until it is no longer sufficient to draw out the newly added wall units. The rate of length extension will fall and a visible septum will develop. The hydrostatic pressure will begin to rise and length extension accelerate again so that the dividing cell will have the characteristic pinched-off appearance that is seen in *E. coli* (e.g. Steed & Murray 1966).

If the doubling in the rate of wall synthesis is delayed by a reduction in replication velocity the internal hydrostatic pressure will rise above the level found at any stage in the cell cycle under steady-state conditions. This will overcome the resistance of the cell to expansion in girth which we assume to be greater than its resistance to expansion in length. The newly added wall units will now tend to become intercalated between existing wall material. This process will continue until the increment in volume for a given increment in length is sufficient to restore to its normal limits the range of hydrostatic pressures experienced by the cell during each cycle of growth and division. The system will be self-regulating in the sense that provided there is a differential resistance to expansion in length and expansion in circumference, and provided that this differential is small, then not only will the cell be rod-shaped with rounded ends under steady-state conditions, but the extent of the change in circumference for a given change in C will be determined (and determinable). Similar considerations will apply during a shift-up because mass increase will accelerate faster than the rate of wall synthesis as a consequence of the progressively decreasing concentration of the rate-limiting wall precursor gene.

In the light of the general conception of the relation between growth, wall synthesis and septum formation presented here we can consider the consequence of interrupting DNA synthesis by thymine starvation or by preventing initiation of new rounds of replication in thermosensitive initiation defective strains.

Thymine starved cultures typically increase in mass exponentially at the prestarvation rate for about one mass doubling after which the rate falls progressively to zero (unpublished data based on the behaviour of many *thy*$^-$ strains). Since the immediate inhibition of DNA synthesis

will prevent any subsequent doubling in the rate of wall synthesis the internal hydrostatic pressure will never fall to the level required to permit septum formation in most cells and cell division will cease (figure 15*b*). In the majority of cells the hydrostatic pressure will rise to an abnormally high level and this may account for the abnormal sensitivity to plating on solid media which is exhibited by thymine starved cells and which has been suggested to be the explanation for 'thymineless death' (Bazill 1967; Donachie & Hobbs 1967; Pinney & Smith 1972), although this interpretation of thymineless death has been challenged (Nakayama & Conch 1973). In this context it is worth noting that sensitivity to 'thymineless death' in *Bacillus subtilis* is reduced by plating starved cells on media of high osmotic strength (Anraku & Landman 1968).

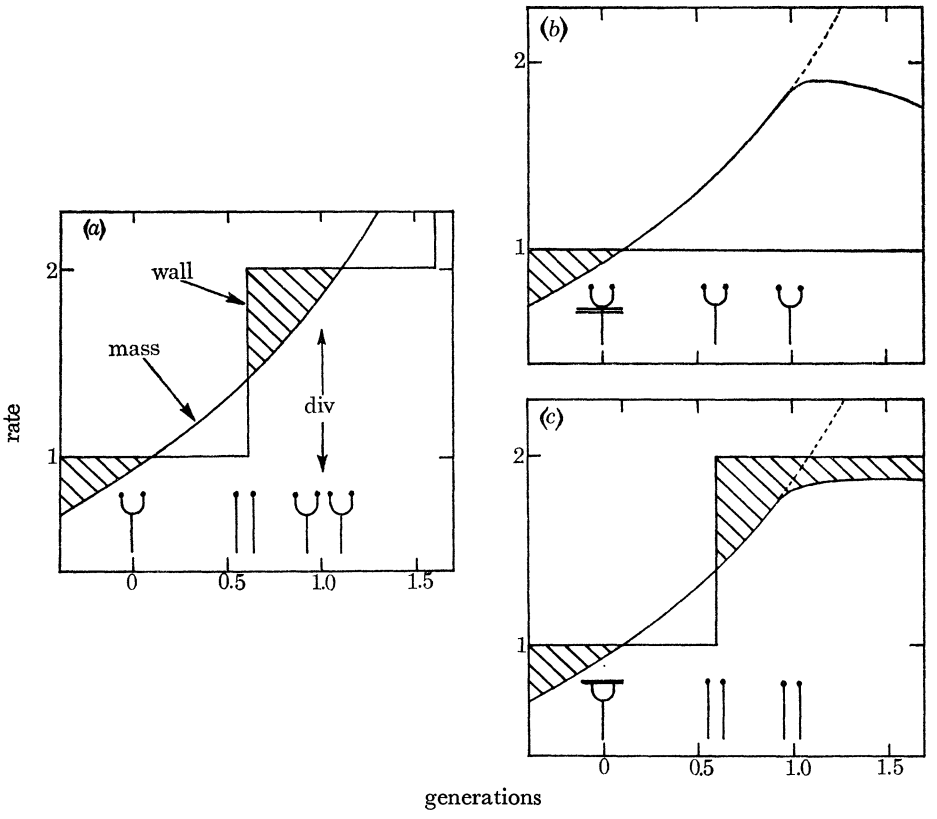

FIGURE 15. A model to illustrate the effect of inhibition of DNA synthesis on cell division. (*a*) The normal cell cycle. The hatched area shows that part of the cell cycle when the rate of wall synthesis exceeds the rate of mass increase. For simplicity the rate of wall synthesis is assumed to double at the time of termination. (*b*) Effect of immediate inhibition of DNA synthesis. Note that the rate of wall synthesis is never in excess. (*c*) Effect of inhibition of initiation of new rounds of replication.

Initiation defective strains also increase in mass exponentially for at least one mass doubling after which the rate becomes linear (unpublished data). Since rounds of replication can be completed, however, the rate of wall synthesis will double in the majority of cells and will therefore be double the rate that would occur in most cells of a thymine-starved culture (figure 15*c*). When the rate of mass increase has become linear the relative rates of wall synthesis and mass increase may therefore be similar to those which occur in a normal cell at the time of septation. Repeated cell division can consequently take place leading to the production of DNA-less cells.

The essence of the model I have outlined is that there is no specific division signal during the

cell cycle and no fundamental difference between cross-wall formation and extension in cell length. Division can occur only when the amount of wall accumulated in a given interval of time is sufficient not only to accommodate the increment in mass during the same period but also lead to a reduction in hydrostatic pressure. The additional mutations which may occur in *thy*$^-$ strains or in mutant strains defective in DNA synthesis and which permit them to continue to divide in the absence of DNA synthesis may be lesions which modify differentially the rates of mass increase and wall synthesis.

The picture of cell division presented here embodies ideas which have been expressed before (Bazill 1967; Previc 1970; Higgins & Shockman 1971). It is clearly an oversimplification of reality. Thus although one prediction arising from the possibility that the gene which effectively determines the rate of wall synthesis is located some distance from the chromosome terminus (figure 13) is that an increase in *C* will lead to a reduction (although not a proportionate reduction) in the time between termination of rounds of replication and cell divsion, as we have found, the rapid response in the rate of cell division to step-up and step-down transitions (e.g. figure 12) is not expected and suggests that termination itself is also involved as originally suggested by Helmstetter *et al.* (1968). Evidence recently obtained by Jones & Donachie (1973) showing that cell division cannot take place if protein synthesis is inhibited at the time of termination of rounds of replication also suggests a specific involvement of termination in cell division. It may well be that the linear-log model for wall cytoplasm synthesis defines the conditions necessary for septum formation to be possible but that termination is needed for the cell to undergo the morphogenetic transition from extension in length to cross-wall formation.

Division defective mutants

Although the many division defective mutant strains of *E. coli* that have been isolated and described will undoubtedly have an important role to play in the future as analytical tools, our present understanding of the lesions involved in such strains is too rudimentary at present for them to throw much light on the control of cell division. On the other hand, no model for cell division can be regarded as satisfactory which does not take account of the properties of strains carrying mutations at a locus variously designated *lon* or *capR* (Howard-Flanders, Simson & Theriot 1964; Markovitz & Baker 1967) in *E. coli* K 12 located at position 10 on the linkage map (Taylor & Trotter 1972) which have been extensively studied over several years.

Lon$^-$ strains differ from *lon*$^+$ strains in that septum formation is abnormally sensitive to treatments which inhibit DNA synthesis and also to a shift-up transition (Howard-Flanders *et al.* 1964; Kantor & Deering 1968; Cummings & Mondale 1967; Walker & Smith 1970). Colony-forming ability also is abnormally sensitive to inhibition of DNA synthesis, but a clear difference in this respect between *lon*$^-$ and *lon*$^+$ strains is generally found only when the assay conditions involve a shift-up (see Howard-Flanders *et al.* 1964; Walker & Smith 1970). Conversely, inhibition of protein synthesis subsequent to inhibition of DNA synthesis tends to abolish the difference between the survival curves and the degree of filamentation of *lon*$^+$ and *lon*$^-$ strains (see Leighton & Donachie 1970). In other words, *lon*$^-$ strains form filaments and lose viability whenever they are subject to 'unbalanced' growth (i.e. to conditions in which the relative rates of mass increase and DNA synthesis differ from those found under steady-state conditions.

The biochemistry of the lesion present in *Lon*$^-$ strains is not understood but they produce

abnormally large amounts of capsular polysaccharide (Markovitz 1964) and tend to form mucoid colonies (Howard-Flanders *et al.* 1964).

On the basis of the model for control of septum formation presented here the behaviour of *lon* mutants would be accounted for if it were supposed that they differ from *lon*$^-$ strains in having a cell wall which is unusually resistant to expansion in girth. This enhanced resistance might be associated with the abnormal amount of capsular polysaccharide produced by *lon* strains, although it has been shown that the mucoid property of these strains can be suppressed by mutations at other loci without altering their sensitivity to unbalanced growth (Donch & Greenberg 1968). The effect of an enhanced resistance to expansion in girth would mean that during shift-up transitions and also when DNA synthesis is inhibited with a consequent reduction in the relative rate of wall synthesis, the cell would compensate for the increased mass to wall ratio by increasing in girth more slowly than normal and would therefore suffer an abnormally high increase in internal hydrostatic pressure. This would prevent septum formation and lead to an enhancement of the plating sensitivity found when *E. coli* is subject to thymine starvation. Inhibition of protein synthesis during or subsequent to inhibition of DNA synthesis would prevent this condition developing and so suppress the Lon phenotype.

The difference in survival between *lon*$^+$ and *lon*$^-$ strains subjected to u.v. irradiation is also suppressed in *exr* and *recA* mutants in which repair of damaged DNA is defective (see Green, Greenberg & Donch 1969). It seems possible that these mutations exert their effect in a quite non-specfic way in that, being repair-deficient, protein synthesis is inhibited to a greater extent by a given dose of irradiation than it is in the corresponding repair proficient strains (this has been shown to be the case by P. T. Barth, personal communication). Thus irradiation of the repair defective strains would act in effect as an inhibitor of protein synthesis.

Bazill (1967) has developed a very similar hypothesis to account for the behaviour of *lon*$^-$ strains. He also assumes that the relative rates of wall synthesis and mass increase vary throughout the cell cycle, the rate of wall synthesis doubling at the time of termination of rounds of replication. He further supposes that rigidity of the outer wall is essential for septum formation to occur (as is also implicit in the model for division outlined here) and that in *lon*$^-$ strains there is a mechanical weakness in the wall which expresses itself only during an inhibition of DNA synthesis. There is little to choose between these two interpretations of the Lon defect but the fact that under identical steady-state conditions the average cell size of *lon*$^+$ and *lon*$^-$ strains is indistinguishable (Walker & Smith 1970) leads us to prefer the notion that in *lon*$^-$ strains the mechanical strength of the outer wall is not abnormally low.

Conclusion

The work which I have covered, in an admittedly speculative and selective rather than comprehensive manner, constitutes the beginnings of a cell biology of *E. coli*. The cell cycle of this bacterium, and probably most others as well, is unusual in that chromosome replication (analogous to the S phase of eukaryotic cells) occupies all or a large part of the doubling time of the cell and at fast growth rates takes longer than one cell generation, in one sense, to complete. The amount of DNA per cell and the relative proportion of different genes are therefore changing continuously both during the cell cycle and at different growth rates. Although this feature of the cell cycle presented an initially confusing problem it can now be seen to provide us with an extremely useful analytical tool for investigating cell growth.

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A revealing aspect of the growth of *E. coli* is the variation in cell size and shape which is associated with variation in the doubling time and the transit time of replication forks. Clearly an important factor which I have not discussed is the chemistry of the cell wall and its biosynthesis. Partly this is because I am not qualified to do so but also it is because I believe that an understanding of the determination of cell size and shape will not be possible without taking into account the physical forces to which the cell boundary is exposed.

It seems unlikely that the cell biology of *E. coli* will be of direct relevance to eukaryotic cells but no doubt the picture as it develops will provide a useful model against which eukaryotic systems can be compared.

The ideas developed in this Review are the outcome of many hours of discussion with my colleagues and students, particularly Shamim Ahmad, Peter Barth, Ifor Beacham, Michael Chandler, John Collins, Peter Meacock and Arieh Zaritsky who have also been responsible for all of the experimental work from this laboratory which has been referred to.

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