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

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

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

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

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

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

RESULTS

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

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

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

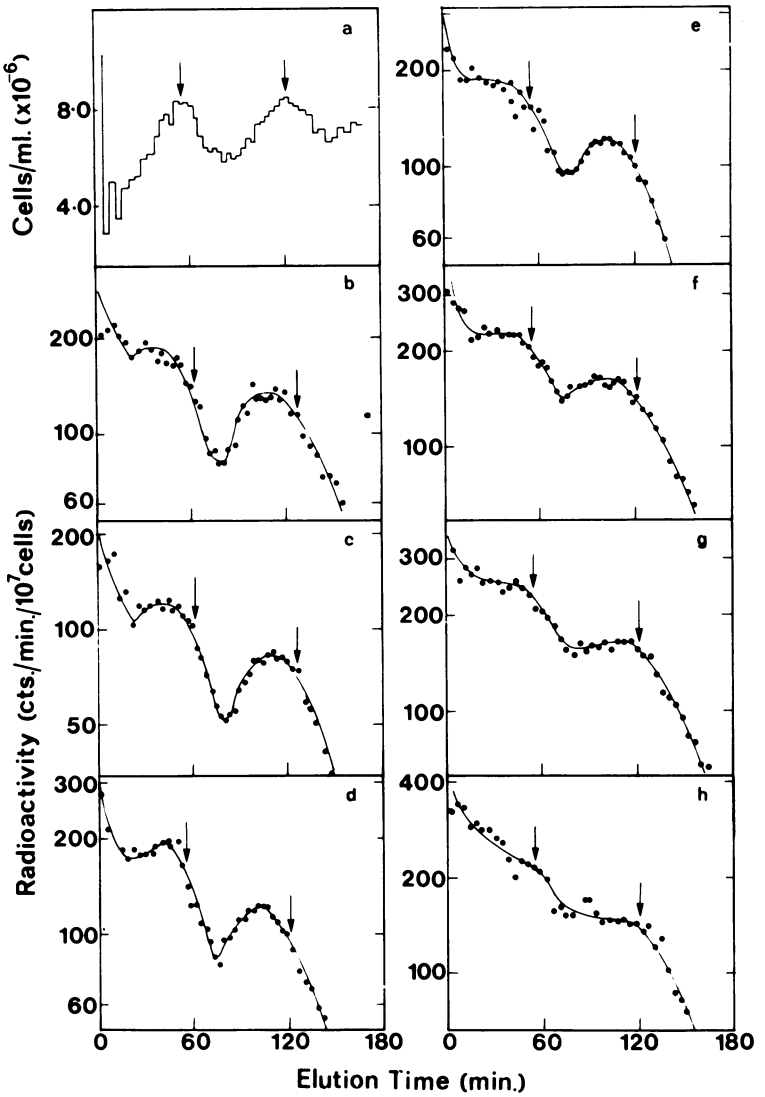

FIG. 1. *Elution patterns of cultures grown in different thymine concentrations. Plots of a typical profile of effluent cell density (a) and radioactivity per effluent cell (b through h) from [^{14}C]thymidine pulse-labeled membrane-bound populations. (b) E. coli B/r Thy$^+$, LEB18; (c, d, e, f, g, h),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

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

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

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

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

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

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

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

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

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

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

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

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

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

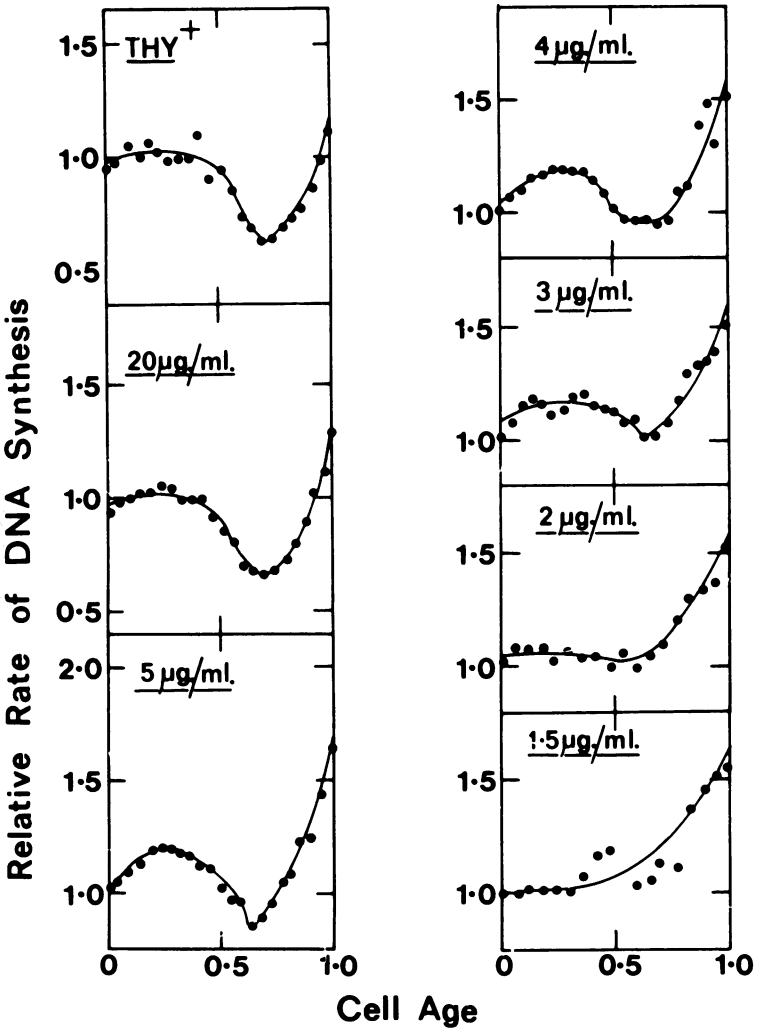

FIG. 2. *Relative rate of DNA synthesis over the cell cycle at different thymine concentrations. The figure is a replot of the curves (b through h) in the second generation of elution in Fig. 1, i.e., the position of the elution curves between the two arrows. The data have been normalized to an initial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

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

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

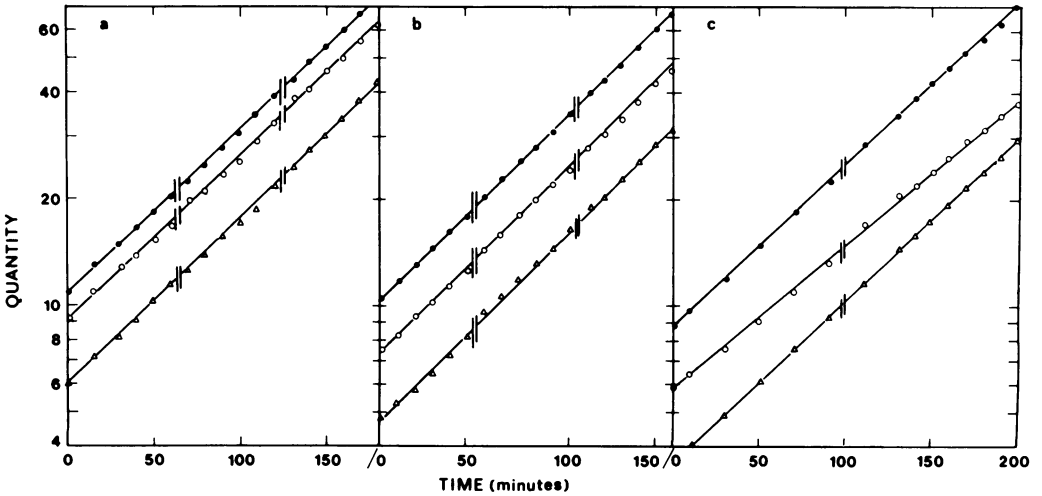

FIG. 3. *Effect of thymine concentration on the rate of increase in absorbance, cell number, and DNA in LEB16 grown in PA medium. A_{450} (10^{-2}) (●), cell number (10^7/ml) (○), and counts per minute (10^3/ml) (△) in cultures uniformly labeled with [^{14}C]thymine (0.05 μCi/μg) at concentrations of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

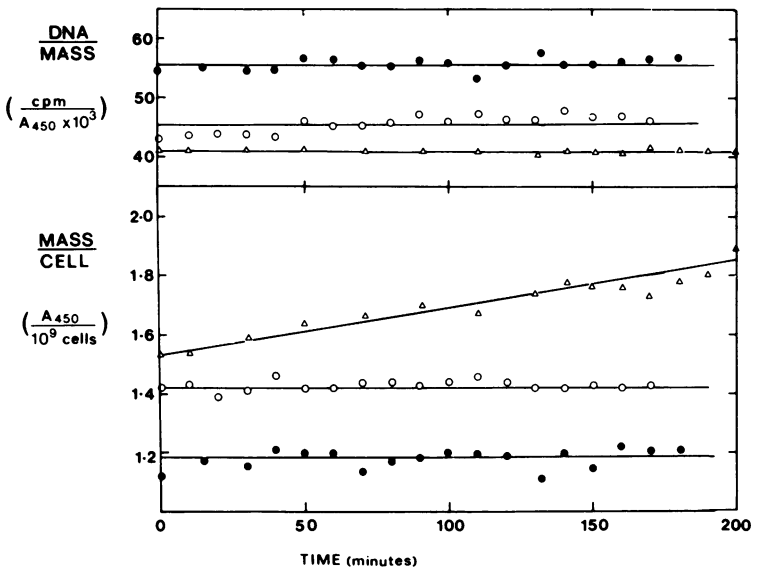

FIG. 4. *DNA-mass ratio ($\bar{G}/\bar{M}$) and average cell mass ($\bar{M}$) for LEB16 grown in PA medium with thymine concentrations of 20 μg/ml (●), 1.5 μg/ml (○), and 1.0 μg/ml (△). The values are calculated from the data in Fig. 3.*

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

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

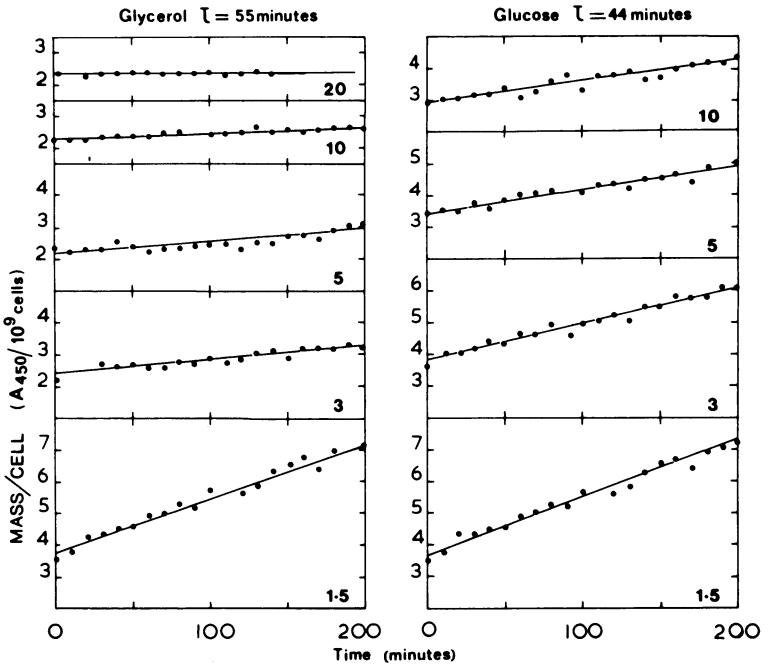

FIG. 5. *Effect of thymine concentration on the rate increase of average cell mass with time of LEB16 grown in media containing glucose and glycerol as carbon source. Thymine concentrations (micrograms per milliliter) are as indicated in the frames.*

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

derived from equation (ii).

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

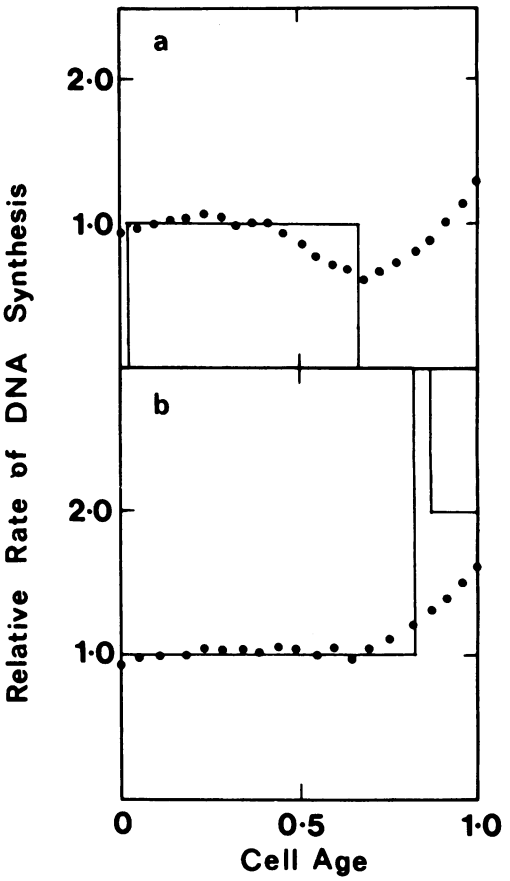

FIG. 6. *Comparison between the observed and predicted patterns of DNA synthesis during growth on high and low thymine. Observed rate of DNA synthesis (●) during the cell cycle for cultures in (a) 20 μg of thymine per ml and (b) 2 μg of thymine per ml (data from Fig. 2). Theoretical rate of DNA synthesis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

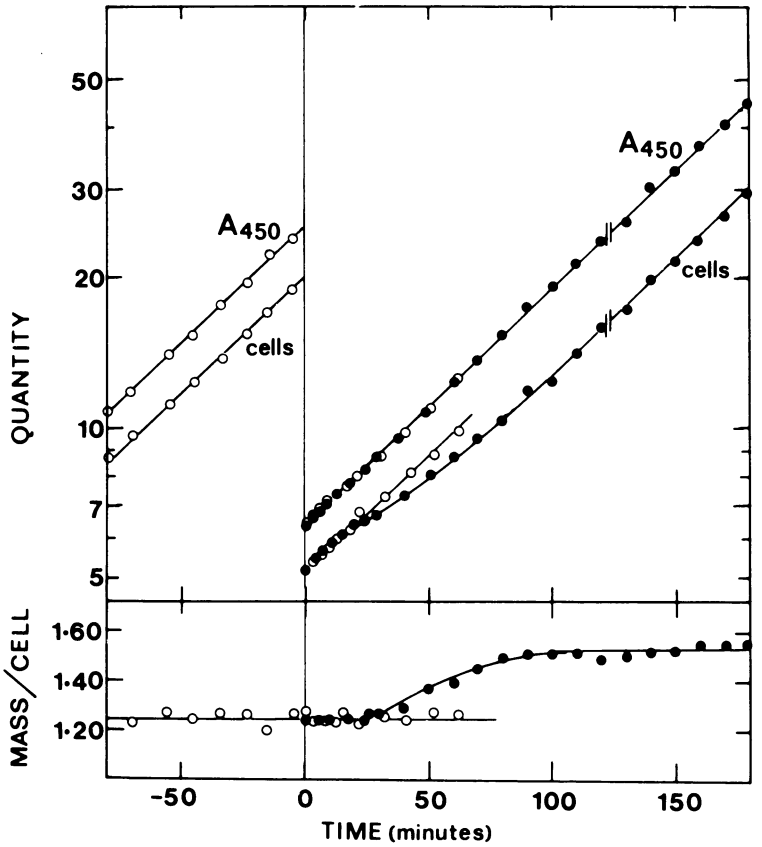

FIG. 7. *The kinetics of increase of absorbance and cell number following a reduction in thymine concentrations. Absorbance A_{450} (10^{-2}) and cell number (10^7/ml) in a culture of LEB16 growing in 6 μg of thymine per ml* (○). *At 0 min the culture was diluted fourfold into fresh prewarmed medium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

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

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

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

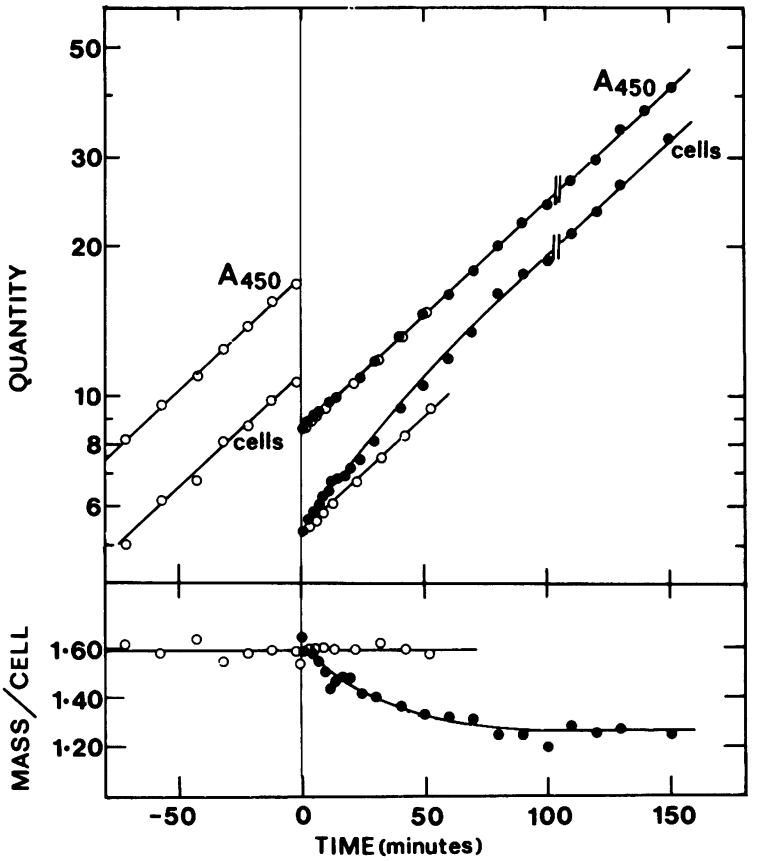

FIG. 8. *The kinetics of increase of absorbance and cell number following an increase in thymine concentration. A_{450} (10^{-2}) and cell number (10^7/ml) in a culture of LEB16 growing in 1.5 μg of thymine per ml (○). At 0 min the culture was diluted twofold into fresh prewarmed medium containing the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

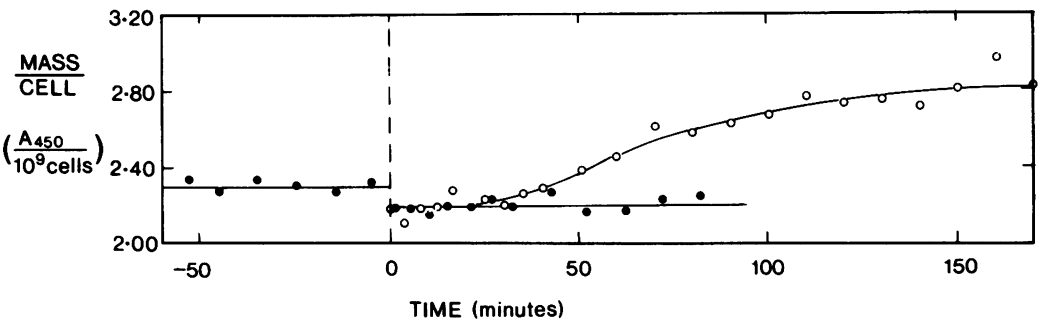

FIG. 9. *Effect of a reduction in thymine concentration on average cell size in E. coli 15T⁻ (555-7) P178. P178 was grown in glycerol medium containing thymine at 2.0 μg/ml (●) and supplemented with required amino acids, arginine, methionine, and tryptophan at 50 μg/ml. At 0 min the culture was diluted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

DISCUSSION

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

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

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

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

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

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