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DNA Synthesis during the Division Cycle of Rapidly Growing *Escherichia coli* B/r

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The rate of DNA synthesis during the division cycle of *Escherichia coli* B/r growing with doubling times between 22 and 40 minutes has been determined by the membrane-selection technique described previously (Helmstetter, 1967). The results indicate that the cell age at which rounds of DNA replication begin is variable and depends upon the growth rate, and that the time for a complete round of replication is constant and independent of the growth rate. It is suggested that in rapidly growing *E. coli* B/r a new round of replication begins before the previous round has ended.

1. Introduction

Evidence has accumulated that replication of the bacterial chromosome begins at a particular site on the chromosome and proceeds sequentially along its entire length (Meselson & Stahl, 1958; Bonhoeffer & Gierer, 1963; Cairns, 1963*a*,*b*; Lark, Repko & Hoffman, 1963; Nagata, 1963; Yoshikawa & Sueoka, 1963*a*,*b*). In most instances the results were consistent with, or demonstrated, a single replication point per chromosome. However, multiple replication points have been reported to occur in the bacterial chromosome during spore germination in *Bacillus subtilis* (Oishi, Yoshikawa & Sueoka, 1964), in rapidly dividing *B. subtilis* (Yoshikawa, O'Sullivan & Sueoka, 1964), and following thymine starvation in *Escherichia coli* (Pritchard & Lark, 1964; Lark & Bird, 1965).

It has been suggested that, at a given temperature, and assuming no substrate or energy limitations, the rate of DNA synthesis per replication point is a constant independent of the over-all cellular growth rate (Maaløe & Kjeldgaard, 1966). One prediction of this hypothesis is that cells which require a longer time to double than the time for a replication point to travel the entire length of the chromosome will have a period devoid of DNA synthesis during their division cycle. The existence of a gap between rounds of DNA synthesis in slow-growing cells has been reported (Lark, 1966; Helmstetter, 1967; Kubitschek, Bendigkeit & Loken, 1967).

A second prediction of this hypothesis is that if cells can double in less time than it takes for a replication point to traverse the chromosome, they will have chromosomes

with multiple replication points for at least part of their division cycle. To investigate this second prediction we have measured the rate of DNA synthesis during the division cycle of *E. coli* B/r in rapidly growing, exponential phase cultures, using a technique previously employed for slow-growing cells (Helmstetter, 1967). Cultures growing exponentially in various media were pulse-labeled with [^{14}C]thymidine and the amount of label incorporated into cells of different ages was determined. The sorting-out of the labeled cells according to age was accomplished by binding the cells to a membrane filter at the end of the labeling period and collecting the new-born cells which were eluted continuously from the membrane. Measurement of the radioactivity in the effluent cells as a function of elution time yielded the rate of thymidine incorporaton into their ancestors as a function of age. In rapidly growing cells, the rate of DNA synthesis during the division cycle followed a pattern which indicated: (a) the presence of chromosomes with multiple replication points; and (b) a constant time for one such point to traverse the entire length of the chromosome.

2. Materials and Methods

(a) *Bacteria and growth conditions*

The organism used was *E. coli* B/r (ATCC 12407). The minimal salts medium contained 2 g NH_4Cl, 6 g Na_2HPO_4, 3 g KH_2PO_4, 3 g NaCl, 0·25 g $MgSO_4$, and 1 g glucose in 1 l. of distilled, demineralized water. The bacteria were grown at different rates by supplementing the basic minimal medium with various amino acid–nucleoside mixtures. The composition of the supplements, and the growth rates obtained, will be described with the results. For each experiment 100 ml. of minimal medium containing glucose as sole carbon source was inoculated with bacteria and incubated approximately 15 hr at 37°C with shaking. At this time the cells were in exponential growth with less than 3×10^6 cells/ml.; supplements were then added and the cultures incubated for at least 4 additional hr until the concentration was 1×10^8 cells/ml.

(b) *Experimental procedure*

The rate of thymidine incorporation during the division cycle of *E. coli* B/r was measured by pulse-labeling the exponential phase cultures with [^{14}C]thymidine (35 mc/m-mole, New England Nuclear Corp.) and determining the amount of label incorporated into cells of different ages. This was accomplished by binding the cells to a type GS Millipore membrane filter (Millipore Corp.) at the end of the labeling period and counting the radioactivity in the new-born cells eluted continuously from the membrane. The binding and elution procedure has been described previously (Helmstetter, 1967). Briefly, after exposure to the label (approximately 1 min) the culture was bound to the membrane by filtration and washed by passing 100 ml. of conditioned medium through the filter. The conditioned medium was composed of 1 part medium which had supported growth to 4×10^8 cells/ml. and had been filtered through a grade HA Millipore filter, and 3 parts fresh medium. The filter assembly was then inverted and elution with conditioned medium begun. Samples of the effluent were collected consecutively during constant time intervals, and the bacterial concentration measured with a Coulter counter model B (see Helmstetter & Cummings, 1963,1964). Each sample was precipitated with cold trichloroacetic acid of 5% final concentration, held in an ice bath for 30 min, collected on 25-mm diameter Whatman glass filter discs, washed with cold 5% trichloroacetic acid containing 100 μg of unlabeled thymidine/ml. and dried. The radioactivity on the discs was determined in Liquifluor (Nuclear Chicago Corp.) with a Nuclear Chicago liquid-scintillation system.

3. Results

(a) *Doubling times of membrane-bound* E. coli *B/r*

The concentration of cells (primarily daughter cells) in the effluent from a membrane-bound population of *E. coli* B/r varies in a periodic manner with elution time. Figure 1

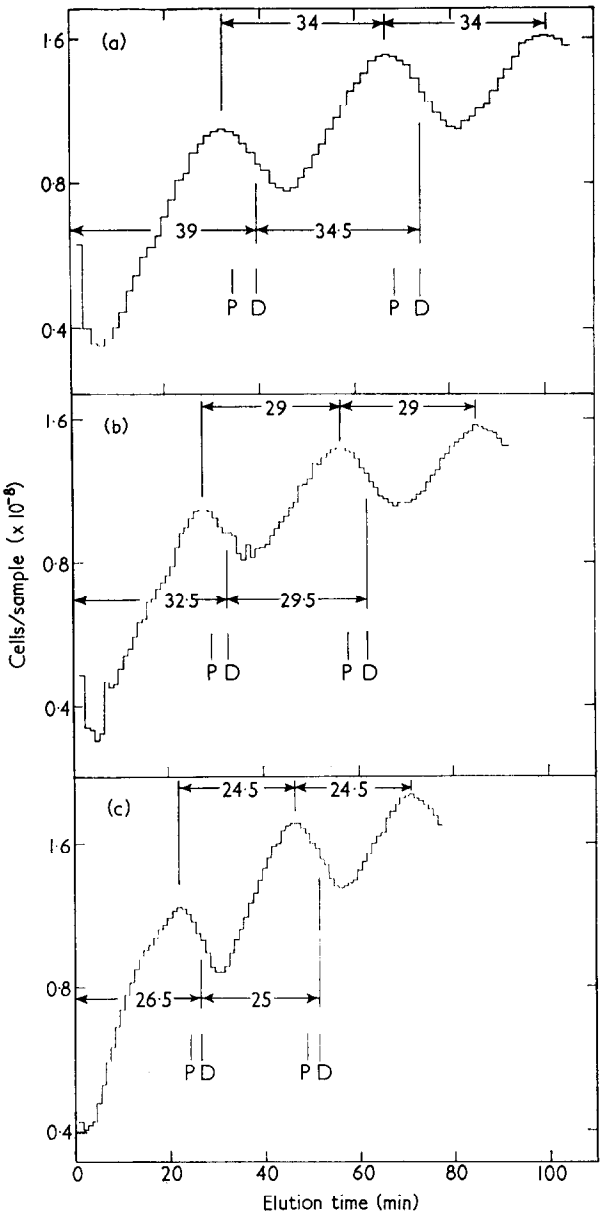

FIG. 1. Elution of cells from membrane-bound populations of *E. coli* B/r.

The number of cells in consecutive samples of the effluent is illustrated. The cells were grown and eluted in glucose minimal medium supplemented with: (a) histidine and arginine, (b) a mixture of 12 amino acids (see Table 1), and (c) Casamino acids and tryptophan. The elution rate and sampling period was 6·5 ml./min and 1·5 min in (a), 9·5 ml./min and 1·0 min in (b), and 9·0 ml./min and 1·0 min in (c). The upper numbers in each diagram are the times between peaks in the elution curve, and the lower numbers show the time between zero and the midpoint of the first decrease in cell number and between the first and second midpoints.

The short vertical lines marked P indicate generations of elution determined as the average time between peaks, marked off as equal intervals from the start of elution. (Note that the time P does not correspond to the positions of the peaks, since the average time between peaks need not correspond to the time between zero and the first peak.) The lines marked D indicate generations of elution determined by the decrease in the elution curves. Each line corresponds to the midpoint of a decrease in concentration. The details of the generation time measurements are described in the text.

shows the concentration of cells in the effluent from membrane-bound populations grown and eluted in glucose minimal medium supplemented with histidine and methionine (Fig. 1(a)), a mixture of 12 amino acids (Fig. 1(b)), and Casamino acids Calbiochem and tryptophan (Fig. 1(c)). The shape of the elution curve in each generation presumably reflects the age distribution of the cells initially bound to the membrane, and it has been assumed that the mean doubling time of the cells growing on the membrane can be determined from the periodicity in the curves (Helmstetter, 1967); e.g. by measuring the average time-interval between peaks in the elution curves. This method for determining mean generation times is valid only if the cells bound to the membrane grow at a constant rate. In general, the time between successive peaks in the elution curves was essentially constant, indicating little, if any, change in growth rate, at least after the first generation. This has been shown in Fig. 1, in which the peak-to-peak measurements of generation times are indicated between the lines marked P.

There are reasons to suspect that the first generation of daughter cells is eluted more slowly than succeeding generations. In an ideal experiment, the first generation could be determined by measuring the time between the beginning of elution and the first abrupt decrease in the concentration of cells in the effluent. Experimentally, the decrease extends over a significant time interval due to the dispersion in generation times of individual cells, but the mean time for the first generation of elution can be estimated by the position of the midpoint of the first period of decrease in concentration. Measured in this way, and compared to the time-intervals between peaks in the same curve, the first generation of elution was usually longer than subsequent generations (Fig. 1).

This observation could mean (a) that the first division cycle on the membrane was excessively long, or (b) that the entire elution curve was shifted to the right because the probability of elution of new-born cells was abnormally low in the beginning. In case (a), the mean generations of elution would coincide with the midpoints of the decreases in cell concentration and the first generation would thus be longer than subsequent ones. The generations of elution determined in this manner are indicated in Fig. 1(a) to (c) by lines marked D. Case (b) would obtain if, initially, a large fraction of the cells which would normally yield progeny into the effluent, divided without release of either of the two daughter cells. This might be expected, since many cells probably bind to the membrane lengthwise such that neither daughter cell would be released at division. In this case, all the cells initially attached would have divided before the first decrease in the elution curve, and the peak-to-peak measurement might therefore be the better estimate of the mean generation time of the cells growing on the membrane.

There is evidence to support both interpretations. The growth rate may have increased with time on the membrane, since the time between peaks in the elution curve was shorter than the doubling time of an exponentially growing culture in the same medium, whereas the time between start of elution and the midpoint of the first decrease in concentration was approximately the same as the logarithmic phase doubling time. A shift in the elution curve is suggested by the observation that the lower the probability of elution of daughter cells, the longer was the time between start of elution and the first decrease. This lower probability was indicated by an extended period of elution of unselected cells at the beginning of elution. In the absence of an independent measurement of generation times on the membrane,

TABLE 1

Determination of doubling times for membrane-bound E. coli *B/r*

Medium‡	Log-phase doubling time† (min)		Time between peaks in elution curves (min)				Time between midpoints of decreases in elution curves (min)			
			1st to 2nd peak		2nd to 3rd peak		0 to 1st midpoint		1st to 2nd midpoint	
0	46	(4)	40	(4)	40	(4)	46	(4)	42	(4)
2	45	(1)	38	(1)	—		42	(1)	39	(1)
1	—		34·5	(1)	35	(1)	40	(1)	35	(1)
2′	40	(1)	34	(1)	34	(1)	39	(1)	34	(1)
3	38	(2)	33·5	(2)	—		36	(2)	34	(2)
6	35	(1)	32	(1)	—		36	(1)	34	(1)
9	37	(1)	31	(2)	31	(1)	34	(2)	33	(2)
12	32	(1)	30	(3)	30	(3)	33	(3)	31	(3)
15	28	(1)	27·5	(2)	27	(2)	30	(2)	28	(1)
18	27	(1)	27	(1)	27	(1)	29	(1)	27	(1)
CAA	25	(1)	24·5	(2)	25	(2)	28	(2)	26	(2)
CAA + UAC	23	(1)	23	(1)	23	(1)	22	(1)	23	(1)
CAA + UAC + UACR	—		22	(1)	21	(1)	21	(1)	22	(1)

The time intervals in the elution curves were determined as described in Fig. 1 and in the text. The numbers in brackets indicate the number of independent determinations. Dashes indicate that the measurement was not made or could not be made.

† The log-phase doubling times were determined on cultures shaking at 37°C using a Coulter counter model B.

‡ The media contained minimal salts with glucose and the following additions:

0, None
2, Histidine, arginine
1, Methionine
2′, Methionine, histidine
3, Methionine, histidine, arginine
6, 3 + proline, leucine, threonine
9, 6 + serine, phenylalanine, tryptophan
12, 9 + cysteine, glycine, isoleucine
15, 12 + aspartic acid, glutamic acid, alanine
18, 15 + tyrosine, asparagine, glutamine
CAA, Casamino acids, tryptophan
CAA + UAC, CAA + uracil, cytosine, adenine
CAA + UAC + UACR, CAA + UAC + uridine, cytidine, adenosine

The final concentrations were: 50 μg/ml. for the amino acids, 0·2% for the Casamino acids and 25 μg/ml. for the bases and nucleosides. Note that the addition of methionine appears to cause an unusually large increase in the growth rate.

they will be calculated by both methods (denoted by P and D) in all of the experiments to be described.

Table 1 shows the composition of the various media and the corresponding times between the peaks and between the midpoints of the decreases in the elution curves. In most cases the peak-to-peak time (or the time between the midpoints of the first and second decrease) is shorter than the time between zero and the first decrease in cell concentration.

(b) *Rate of thymidine incorporation during the division cycle*

The rate of thymidine incorporation into cells of different ages in populations of *E. coli* B/r was determined by pulse-labeling the cells with [^{14}C]thymidine, binding the labeled cells to a membrane filter, and counting the radioactivity in the new-born cells eluted continuously from the membrane. Figure 2(a) shows the radioactivity

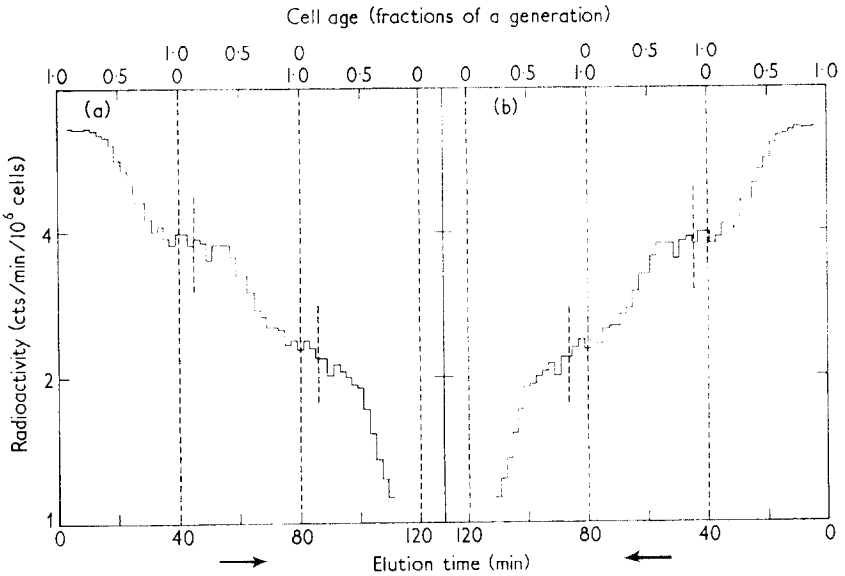

FIG. 2. Radioactivity per cell in the effluent from a membrane-bound culture of *E. coli* B/r.

Cells growing in minimal medium with glucose as sole carbon source were exposed to 0·02 μc/ml. of [^{14}C]thymidine for 2 min, bound to a membrane filter and eluted with glucose minimal medium.

(a) Radioactivity per cell plotted logarithmically as the experimental data were obtained with elution time increasing from left to right. The vertical, dashed lines extending the length of the Figure correspond to line P, and the short, dashed lines to line D in Fig. 1. At the top of the frame the ages of the ancestors of the eluted cells at the time of labeling are shown. Cell age in this and the remaining Figures was determined using the peak to peak (P) measurements of generations as described in the text.

(b) The same data as (a) plotted in reverse so that elution time increases from right to left. In this case the cell age increases from left to right (top) in each generation, so that the relative rate of thymidine incorporation during the division cycle is given directly in each generation. The elution rate was 4·3 ml./min and the sampling period, indicated by the horizontal lines in the histograms, was 2 min.

per cell during elution of a pulse-labeled, glucose-grown culture. The dashed lines which extend the length of the Figure indicate generations of elution based on average time between peaks in the elution curve, and the short dashed lines indicate generations estimated from the midpoints of the decreases in bacterial concentration in the same curve. Since the cells eluted first were progeny of the oldest cells initially attached to the membrane, and those eluted at one generation were progeny of the youngest cells, the relative rate of thymidine incorporation, as a function of increasing cell age, can be read from right to left in each generation. In Fig. 2(b) the same data have been plotted in reverse so that cell age increases from left to right in each generation. Between the pairs of vertical lines it can be seen directly that the rate of thymidine incorporation increased around the middle of the cell cycle in glucose-grown cells, as was previously reported (Helmstetter, 1967). Similar results for glucose-grown *E. coli* B/r were obtained by Clark & Maaløe (1967).

Figure 3 shows the radioactivity per effluent cell during elution of cells grown and pulse-labeled with [^{14}C]thymidine in various enriched media. In each case the rate of thymidine incorporation increased sharply during a portion of the cell cycle, and in some of the rapidly growing cultures (Fig. 3(c), (d) and (e)) the rate decreased again shortly after the increase. We shall assume that the time in the division cycle when the rate of thymidine incorporation increases in the average cell can be estimated

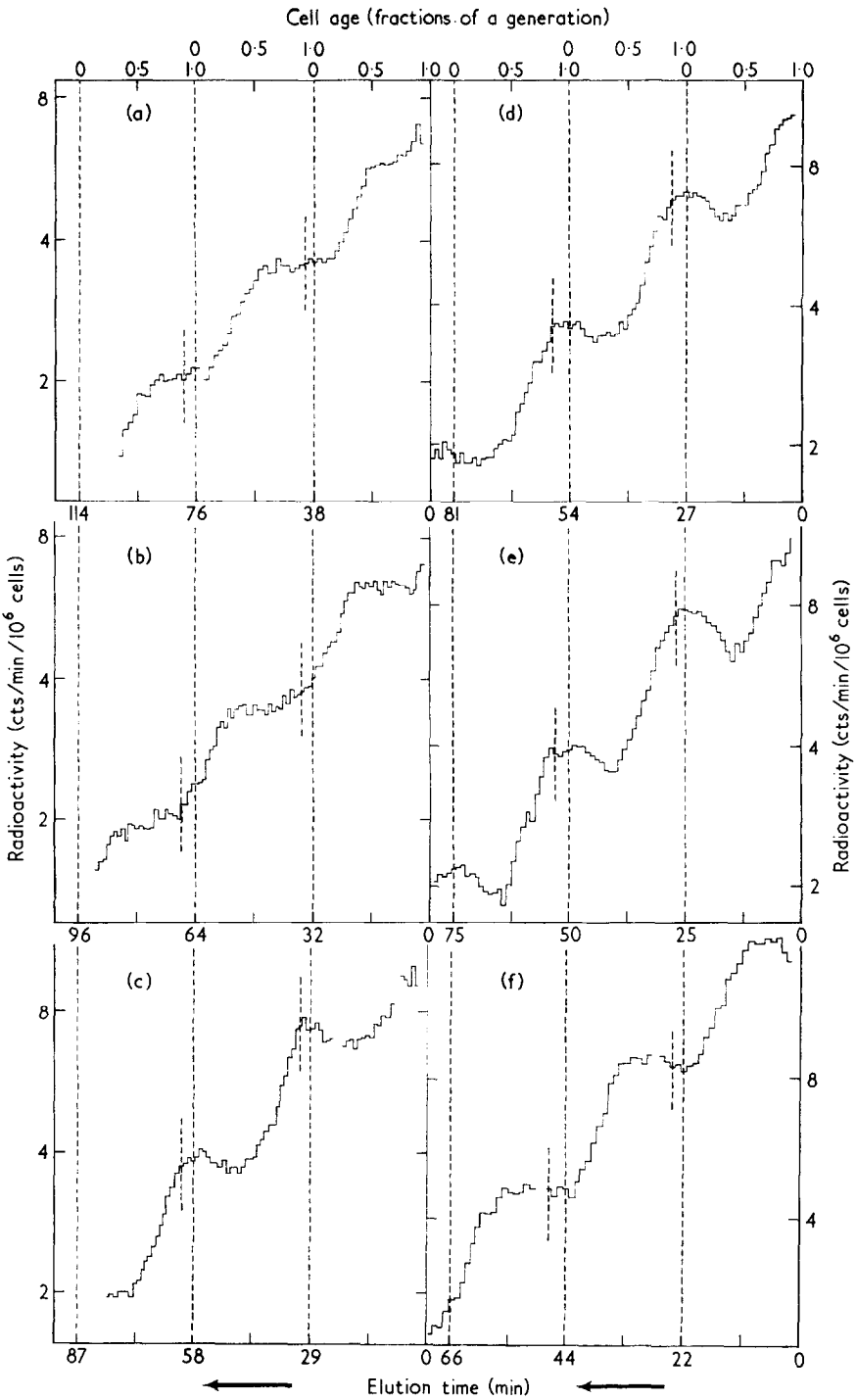

FIG. 3. Radioactivity per cell in the effluent from membrane-bound cultures growing at different rates.

The curves are plotted as in Fig. 2(b), so that the rate of [^{14}C]thymidine incorporation as a function of increasing cell age can be read from left to right in each generation. The cells were grown and eluted in glucose minimal medium supplemented with: (a) 2 amino acids; (b) 6 amino acids; (c) 12 amino acids; (d) 15 amino acids; (e) CAA; (f) CAA + UAC + UACR. The compositions of the supplements are given in Table 1. The cell age shown at the top is based on the peak-to-peak measurements of generation times (see text). In each experiment 100 ml. of cells at 1×10^8 cells/ml. were exposed to 0·02 μc/ml. of [^{14}C]thymidine for 1 min. The elution rate was 6·5 ml./min in (a); 9·5 ml./min in (b) to (e); and 9·0 ml./min in (f). The sampling period was 1·5 min in (a) and 1·0 min in (b) to (f).

by determining the cell age which corresponds to the midpoint of the increase in radioactivity per effluent cell. The time of the increase in radioactivity per effluent cell shifts to the left beginning in Fig. 2(b) and continuing in Fig. 3(a) to (f). In cells grown in minimal-salts medium containing glucose (Fig. 2(b)), glucose plus arginine (Fig. 3(a)), or glucose plus a mixture of six amino acids (Fig. 3(b)), the rate of thymidine incorporation increased in a simple step-wise fashion during the division cycle. In the more rapidly growing cells (Fig. 3(c), (d) and (e)), each increase in the rate was followed by a marked decrease. At the highest growth rate shown (Fig. 3(f)), the decrease is barely visible.

The results shown in Fig. 3 are representative of all the experiments listed in Table 1, and they indicate that the rate of thymidine incorporation increased at progressively earlier times with reference to a particular division, as the growth rate increased. Figure 4 shows the cell age at the midpoint of the increase as a function of

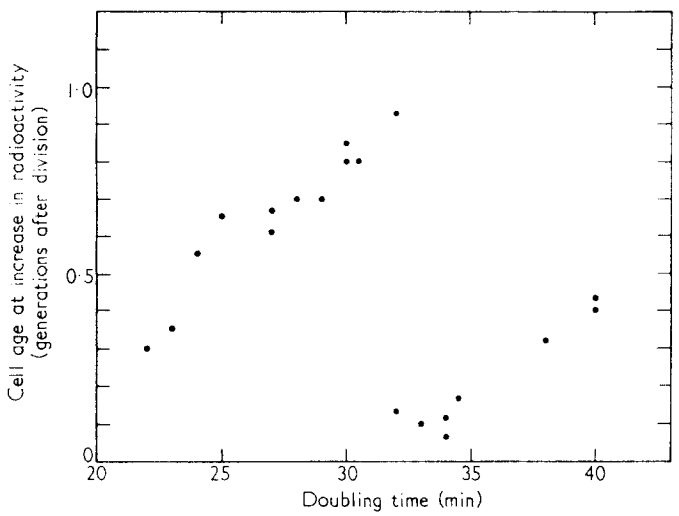

FIG. 4. Cell age at the time of increase in rate of DNA synthesis *versus* growth rate.

In each of the experiments listed in Table 1, the rate of [^{14}C]thymidine incorporation was determined as shown in Fig. 3. In this Figure the average age at the midpoint of the increase in radioactivity per cell in each of these experiments is plotted *versus* doubling time. The cell ages and the doubling times were determined by the peak-to-peak measurement of generation times (see text).

doubling time for the experiments listed in Table 1. Between growth rates of 40 and 32 minutes per doubling, the rate of incorporation increased earlier in the division cycle with increasing growth rate. At a doubling time of about 32 minutes, there was a sudden shift in the time at which the rate increased from early to late in the division cycle. With further increases in growth rate from 32 to 22 minutes per doubling, the the increase in rate occurred in younger cells.

4. Discussion

(a) *Interpretation and quantitative analysis of increases and decreases in the rate of thymidine incorporation*

The rate of [^{14}C]thymidine incorporation during the division cycle of *E. coli* B/r has been determined in cultures growing exponentially in media which supported

growth at rates between 22 and 40 minutes per doubling. One fairly abrupt increase in this rate was always observed during the division cycle, and at high growth rates a decrease was also noted. These abrupt changes could reflect increases and decreases in the number of DNA replication points in a cell, or they could be due, at least in part, to changes in the rate of DNA synthesis at individual replication points.

A number of observations suggest that the increases and decreases in the rate of DNA synthesis can be caused by changes in the number of replication points. The autoradiographic data of Cairns (1963*a*) are consistent with a constant rate of DNA synthesis at a replication point in *E. coli* growing with a 30-minute doubling time. Clark & Maaløe (1967) have shown that a new round of DNA replication is initiated at the time the rate of DNA synthesis increases in glucose-grown *E. coli* B/r. The dramatic decrease and subsequent increase in thymidine incorporation, which suggests a gap in the synthesis of DNA in slow-growing cells, may reflect the disappearance and reappearance of replication points (Helmstetter, 1967). Forro & Wertheimer (1960) have presented direct evidence using autoradiographic techniques that rounds of replication can begin in the middle of the division cycle.

For these reasons we assume that an increase in the rate of DNA synthesis corresponds to the start, and a decrease to the end of a round of DNA replication. A detailed model which accounts for the number of genomes involved in each round of replication is presented in the following paper (Cooper & Helmstetter, 1968). In cells in which both an increase and a decrease are observed, we conclude that *rounds of replication begin and end at different times during the division cycle.*

It has previously been suggested that in slow-growing cells of *E. coli* B/r with doubling times between 50 and 100 minutes, there is a delay between the end of one round and the start of the next round of replication (Helmstetter, 1967). The present experiments indicate that the start and finish of rounds are also out of phase in cells with doubling times between 24 and 30 minutes. We suggest that, in contrast to slow-growing cells, the start and end of rounds are out of phase in these rapidly growing cells because a new round of replication begins before the previous one has ended. Consequently, these cells contain more than one replication point per chromosome between the time a new round begins and the previous round ends.

Multiple replication points in individual bacterial chromosomes have previously been observed in germinating *B. subtilis* spores (Oishi *et al.*, 1964), in fast-growing vegetative cells of *B. subtilis* (Yoshikawa *et al.*, 1964) and following thymine starvation in *E. coli* (Pritchard & Lark, 1964). The pattern of DNA synthesis reported here is consistent with the occurrence of multiple forks during a portion of the division cycle of rapidly growing *E. coli* cells. There is strong evidence that each new round of replication begins at a defined point on the bacterial chromosome and always proceeds in the same direction, at least in a given strain (see Introduction). In a chromosome which already contains one replication fork, the initiation of new rounds of replication, one in each arm of the fork, would increase the rate of DNA synthesis from one to three, assuming a fixed rate of synthesis per replication point, When the original, single replication point reaches the end of the chromosome, the rate of DNA synthesis would decrease from three to two. These changes would be observed as a peak in the rate of DNA synthesis during the period of multiple forks. This "peaked" pattern is evident in Fig. 3(c), (d) and (e).

The over-all pattern of DNA synthesis is thus consistent with the concept of multiple forks, but the increases and decreases in rate are not as large as predicted.

In cells in which the rise and fall were about one-half generation apart (Fig. 3(e)) the rate of DNA synthesis increased by a factor of 2·3 against the theoretical factor of 3; in the other cases the observed increases were about twofold. However, dispersion in the release of new-born cells would tend to decrease the magnitude of the changes in rate, especially in the experiments with diphasic incorporation patterns. Therefore, we have based our interpretation of these experiments more on the distinctive shape of the curves than on absolute values.

In cells with doubling times between 30 and 40 minutes, essentially no decrease in the rate of DNA synthesis was observed during the division cycle. This could mean either that a new round of DNA replication began the moment a previous round ended, or that the time interval between these two events was too small to be detected. We consider the latter explanation the more plausible, and believe that coincidence of the start and end of rounds is a rare event (see the model proposed in the following paper). In fact, the plateaux in the incorporation curves become flatter with increasing growth rate as if a short peak at the end of the plateaux has been masked by dispersion. Again, at the highest growth rate studied (Fig. 3(f)), no peak in the thymidine incorporation curve was observed, presumably because multiple forks are present most of the time and the brief period of decreased rate was obscured by dispersion. At an even higher growth rate, the diphasic incorporation pattern would be expected to disappear since, if the rate of movement of a replication point is constant, multiple forks would be present throughout the division cycle when the generation time equals one-half the time required for a replication point to traverse the chromosome.

(b) *Continuous variation in the start of rounds of replication*

We have assumed that an increase in the rate of DNA synthesis indicates the start of a round of replication. The cell age at which this happens can therefore be read from Fig. 4, which shows that rounds began earlier in the cell cycle as the growth rate increased, with a discontinuity at a rate of 32 minutes per doubling. This discontinuity is deceiving since, according to our interpretation, the initiation time is displaced in a continuous manner with increasing growth rate.

This is best illustrated if the relationship between the start of rounds and the division cycle is described in slightly different terms: we wish to specify the time in the division cycle at which rounds start in terms of fractions of a generation *before* a division rather than in terms of cell age (i.e., fractions of a generation *after* division). Specifically, *the age at which a round starts will be defined in terms of fractions of a generation before the end of the division cycle in which that round is completed.* For example, in a culture with a doubling time of 40 minutes, rounds of replication begin at age 0·4 and end after the cells subsequently divide. In this case rounds start 0·6 generation before the cells divide and 1·6 generations before the division at the end of the cycle in which the round is completed. In rapidly growing cells, the round which starts at a given increase in the rate of DNA synthesis ends at the second subsequent decrease in rate since the first decrease corresponds to the end of the previous round. Thus, in cells growing in glucose plus Casamino acids, rounds start at age 0·6 and end in the second division cycle after the cycle of initiation (Fig. 3(e)). In this case a round starts at 2·4 generations before the end of the cycle in which it is completed. The advantage of this terminology is that the continuous variation of the time of initiation with increasing growth rate is immediately apparent. This relationship is illustrated in Fig. 5.

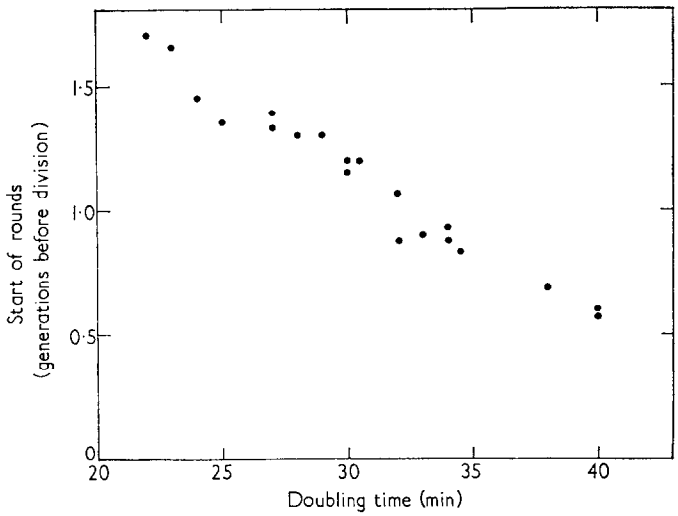

FIG. 5. Cell age at the start of rounds of DNA replication, as a function of growth rate.

Start of rounds (as determined by an increase in rate of DNA synthesis) is plotted in terms of fractions of a generation prior to the end of the division cycle in which the round is completed. See text for details.

(c) *Time for a round of DNA replication*

When both the beginning and the end of a round of DNA replication were observed by an increase and a decrease, respectively, in the rate of DNA synthesis, the absolute time for one round of replication can be estimated as the time between the midpoint of one increase and the midpoint of the second subsequent decrease in the rate of synthesis. Measured in this way, the average time for a round of replication was 41 minutes for doubling times between 23 and 30 minutes (see e.g. Fig. 3(d) and (e)). This is probably a minimum estimate since, as noted above, the doubling time on the membrane may be shorter than the log-phase doubling time. If the log-phase doubling time is used in the calculation, the replication time would be 46 minutes. In cells in which the rate of synthesis did not decrease during the division cycle, either the start and finish of rounds occurred simultaneously or they were slightly out of phase. It would be of interest to know at what growth rate these events coincide during the division cycle. This must occur in cells with a doubling time between 50 and 30 minutes, since in cells growing more slowly than 50 minutes per doubling a gap between rounds has been observed (Helmstetter, 1967), and at doubling times shorter than 30 minutes multiple forks were evident. In recent experiments with glycerol-grown cells, with doubling times of about 50 minutes, the average time for a round of replication was estimated at 42 minutes by measuring the time between the start of a round and the beginning of the gap in DNA synthesis (Cooper & Helmstetter, 1968). From this it appears that the hypothesis of a constant time for a round of DNA replication suggested by Maaløe & Kjeldgaard (1966) is correct in the range of growth rates investigated. At slower growth rates (longer than about 70 minutes per doubling), the time for a round of replication is greater than 41 minutes (Lark, 1966; Helmstetter, 1967). This increase in time is presumably due to limitations of precursors or energy in cells growing with poorer carbon sources.

(d) *Conclusions*

Our measurements of the rate of thymidine incorporation during the division cycle of *E. coli* B/r growing at rates between 22 and 40 minutes per doubling indicate the following:

(1) The cell age at the start of rounds of DNA replication varies with the growth rate. With increasing growth rate, rounds start at increasing fractions of a generation before a given division.

(2) The time for a complete round of replication is quite constant and equals about 41 minutes.

(3) In rapidly growing *E. coli* B/r, a new round of replication begins before the previous round has ended; that is, individual chromosomes contain multiple forks.

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