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# Deoxyribonucleic Acid Synthesis During the Division Cycle of *Escherichia coli*: a Comparison of Strains B/r, K-12, 15, and 15T⁻ Under Conditions of Slow Growth

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The rates of deoxyribonucleic acid (DNA) synthesis during the division cycles of the *Escherichia coli* strains B/r, K-12 3000, 15T⁻, and 15 have been measured in synchronous cultures, under several conditions of slow growth. These synchronous cultures were obtained by sucrose gradient centrifugation of exponentially growing cultures, after which the smallest cells were removed from the gradient and allowed to grow. Sucrose gradient centrifugation did not adversely affect the cell cycle, since an experiment in which an exponentially growing culture was pulsed with [$^3$H]thymidine prior to the periodic separation and assay of the smallest cells resulted in the same conclusions, as given below. In the strains of *E. coli* that were studied, a decreased rate of [$^3$H]thymidine incorporation was seen late in the cell cycle, prior to cell division. No decrease in the rate of [$^3$H]thymidine incorporation was seen at or near the beginning of the cell cycle. Thus, all these strains appear to regulate DNA synthesis in a similar fashion during slow growth. In addition, a correlation between the appearance of cells with visible cross-walls and the start of a new round of DNA synthesis was seen, indicating that these two events might be related.

A conflict exists in the literature regarding the relationship between the timing of deoxyribonucleic acid (DNA) synthesis and the cell division cycle of slowly growing *Escherichia coli*. Different conclusions have been reached when different strains and techniques were used (1, 2, 4, 5, 8, 11, 12, 15-17).

Helmstetter has found that a gap in the DNA synthesis exists only during the last one-third of the cell cycle of *E. coli* B/r by using the membrane elution technique (5, 11). Other workers, using *E. coli* 15T⁻ and *E. coli* B/r, have evidence for a gap in DNA synthesis at or near the beginning of the cell cycle (2, 15-17).

Therefore, we compared DNA synthesis in the *E. coli* strains B/r, K-12, 15, and 15T⁻ under the conditions of slow growth supported by poor carbon sources such as aspartate, acetate, succinate, and proline. Sucrose gradient centrifugation was used to obtain synchronous cultures of the four strains; the membrane elution technique (13) cannot easily be applied to other strains of *E. coli* (6, 11). By using these synchronous cultures, we measured [$^3$H]thymidine uptake throughout the cell cycle. We also determined the time during the cell cycle at which cells with visible cross-walls appeared, in order to study the relationship between DNA initiation and septum formation in these cells. Clark (3), using glucose-grown B/r/1 cells, reported a period of 5 to 7 min between cell cross-wall formation and actual physical separation of the two daughter cells (T period). We measured the T period under conditions of slow growth.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *E. coli* strains B/r, B/r Thy⁻, K-12 3000, 15 (ATCC 9723), and 15T⁻ (ATCC 9723H) were used. Strains B/r and K-12 3000 are from the collection of this laboratory. The bacteria were grown aerobically in a gyratory shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 37 ± 0.50 C in a modified M-9 medium (7 g of $Na_2HPO_4$, 3 g of $KH_2PO_4$, 0.5 g of NaCl, 1 g of $NH_4Cl$, 0.2 g of $MgSO_4 \cdot 7HOH$ per liter of distilled water). Sodium succinate, sodium acetate, sodium aspartate, or proline were added as carbon sources at 1 g/liter. Thymine, required by the *E. coli* strain 15T⁻, was supplied at a concentration of either 10 or 50 μg/ml. When [$^3$H]thymine was used, cells were grown in 2 μg of nonradioactive thymine per ml. Growth of the cultures was monitored with a Klett-Summerson colorimeter fitted with a blue filter.

**Sucrose gradient synchrony.** The method used for sucrose gradient synchrony was a modification of the procedure described by Mitchison and Vincent (18). An overnight stationary culture was diluted into fresh medium containing the appropriate carbon source

and allowed to grow for 5 to 6 generations to approximately $3 \times 10^8$ cells/ml. Cells (10 to 20 ml) were centrifuged at $3,000 \times g$ for 5 min in a Lourdes model AX centrifuge. The pellet was resuspended in 0.5 ml of preconditioned medium that was prepared on the same day by growing cells to $10^8$ cells/ml and removing the cells with a 0.45-$\mu$m membrane filter (Millipore Corp., Bedford, Mass.). The cells were then layered in a glass tube (2 by 14.5 cm) onto a 40-ml 5 to 30% linear gradient of sucrose dissolved in M-9 medium plus the carbon source; the cells were centrifuged at 3,400 rpm for 8 min in an International size 2 swinging-bucket centrifuge at room temperature (25 C). A turbid band of cells was observed approximately one-third of the distance down the tube. Approximately 1.0 ml was removed from the top of this band (less than 4% of the cells) by means of a syringe tip fitted to a polystaltic pump (Buchler Instruments) and added to 20 to 30 ml of fresh medium at 37 C. Very similar results were obtained by using preconditioned medium. The entire centrifugation procedure took approximately 15 to 20 min.

**Counting cell numbers.** Samples were immediately chilled and fixed with 5% formaldehyde. Samples could be kept at 0 C for 5 to 6 days with no detectable change in cell number. For each determination, a minimum of 500 cells was counted in a Petroff-Hauser counting chamber using a phase-contrast microscope with 400-fold magnification. Duplicate counts (300 to 500 per sample) were within 5 to 8%. Dividing cells in which the incipient septa could be seen were counted as one cell. Cells were counted as septated cells from when they showed a central "waist" to when they appeared as pairs of separately distinct but joined cells.

**Isotope incorporation.** Incorporation of [$^3$H]thymidine (specific activity 20 Ci/mmol) or [$^3$H]thymine (specific activity 20 Ci/mmol, New England Nuclear Corp., Boston, Mass.) into trichloroacetic acid-insoluble material was used to measure DNA synthesis. For the pulse experiments, a solution containing 1.2 $\mu$g (1.0 mCi in 12 $\mu$g) of the isotope in M-9 medium per ml was prepared. For each pulse, 0.4 ml of culture was added to 0.1 ml of isotope solution, and the sample was incubated for either 3 or 6 min at 37 C. Incorporation was shown to be linear over at least a 15-min interval after the addition of the isotope. The trichloroacetic acid-precipitable activity was determined by adding samples to 2.3-cm Whatman filter paper disks; the disks were then soaked in 5% trichloroacetic acid and dried, and the acid was removed with five acetone washes. The radioactivity was counted in a liquid scintillation system [toluene-1,4-bis-(5-phenyloxazolyl)benzene]. All experiments were repeated at least three times with similar results.

## RESULTS

**Pattern of DNA synthesis during the division cycle of E. coli B/r.** The doubling time of *E. coli* B/r grown in 0.1% acetate was 93 to 95 min. The sucrose synchronization procedure gave sharp steps of increases in cell number, indicating that this was an effective method for obtaining synchrony. The rate of [$^3$H]thymidine incorporation remained constant for the first 60 min of the division cycle, followed by a 25-min period of decreased [$^3$H]thymidine incorporation (Fig. 1, and summarized in Table 1). This decrease in [$^3$H]thymidine incorporation was striking; there was no incorporation above background level in the last one-third of the first cell division cycle after synchrony was achieved. At a cell age, in fractions of a generation, of 0.90, the rate of [$^3$H]thymidine incorporation increased to double the rate in the first two-thirds of the first cycle. The time during the cell cycle which corresponded to the midpoint of the increase in the radioactivity curve was taken as the cell age at initiation of chromosome replication. The point at which one cell cycle ends and the next begins was taken as the midpoint of the increase in the cell number curve.

Progressive cycles were less synchronized than the first cycle, although the same general pattern of incorporation was seen. No decrease in [$^3$H]thymidine incorporation was observed at the beginning of any cell division cycle.

*E. coli* B/r cells grown in 0.1% succinate had a generation time of 85 min in the first cell cycle after sucrose synchronization and a generation time of 65 min in the second cell cycle. There

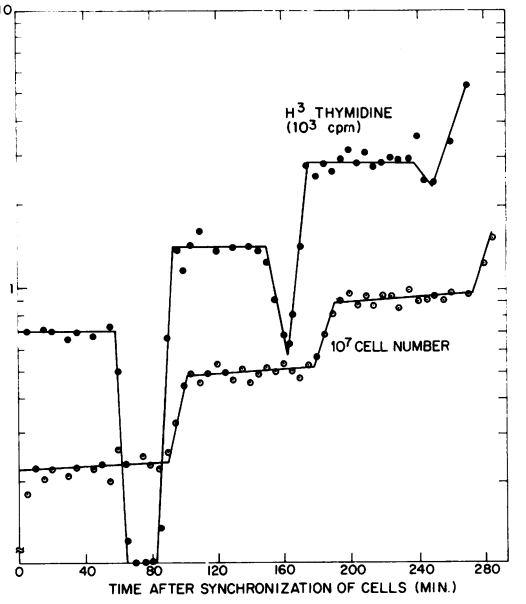


FIG. 1. *Rate of [$^3$H]thymidine incorporation in a culture of E. coli B/r synchronized by sucrose gradient centrifugation. Cells were grown in 0.1% acetate at 37 C. Three cell cycles are shown. [$^3$H]thymidine pulses (●); cell number (⊙).*

TABLE 1. *DNA synthesis during the cell division cycle*[a]

| Time (min) | Succinate | | | | Acetate | | | | Aspartate | Proline |
|---|---|---|---|---|---|---|---|---|---|---|
| | B/r | K-12 | 15 | 15T⁻ | B/r | K-12 | 15 | 15T⁻ | 15T⁻ | 15T⁻ |
| Generation time[b] | | | | | | | | | | |
| First generation | 85 | 72 | 62 | 76 | 95 | 103 | 75 | 70 | 115–120 | 160–165 |
| Second generation (C + D) | 65 | 74 | 50 | 76 | 93 | 100 | 62 | 65 | | |
| Time of DNA initiation (first generation)[c] | 73 | 70 | 46 | 66 | 86 | 90 | 62 | 62 | | 155 |
| Peak % of septated cells[d] | 75 | 64 | 48 | 64 | 85 | 88 | 65 | 60 | 100<br>105 | 150–155 |
| Length of T period[e] | 10 | 7–9 | 14 | 10–13 | 10 | 10 | 10 | 10 | 10–15 | 5–15 |
| C period[f] | 48 | 48 | 36 | 42 | 62 | 62 | 47 | 40 | 83 | 120 |
| D period[g] | 37 | 24 | 26 | 34 | 33 | 41 | 28 | 30 | 32 | 40–45 |
| Age at initiation[h] | 0.85 | 0.96 | 0.74 | 0.86 | 0.90 | 0.87 | 0.82 | 0.88 | 0.95 | 0.96 |
| C/(C + D) | 0.56 | 0.67 | 0.58 | 0.55 | 0.65 | 0.60 | 0.63 | 0.57 | 0.72 | 0.73 |
| D/(C + D) | 0.44 | 0.33 | 0.42 | 0.45 | 0.35 | 0.40 | 0.37 | 0.43 | 0.28 | 0.27 |

[a] The numbers in this table are the average of three experiments. The numbers refer to the first generation after synchronization unless otherwise indicated, since calculations for succeeding generations become less precise due to the increasing amount of asynchrony in the population.
[b] Calculated from midpoint of increase in cell number.
[c] Calculated from midpoint of increase in [$^3$H]thymidine.
[d] Time in the cell cycle at which peak occurred.
[e] Time from peak of septated cells to midpoint in cell number increase.
[f] Period of DNA synthesis.
[g] Period between end of DNA synthesis and cell division.
[h] In fractions of a generation.

was a decrease in [$^3$H]thymidine incorporation late in both the first and second cell cycles, between the cell ages of 0.57 to 0.85 (Fig. 2). The length of the gap in DNA synthesis was determined by measuring the length of time between the midpoint of the decrease in [$^3$H]thymidine incorporation and the midpoint of the increase in [$^3$H]thymidine incorporation.

Initiation of a new round of DNA replication occurred 12 min prior to the end of the first cell division cycle. The increase in the rate of [$^3$H]thymidine incorporation over the first 15 min of the first cycle was presumably due to the synchronization procedure, since it was not seen in the next two cell cycles, in which the rate of DNA synthesis doubled late in the cell cycle. When a succinate-grown *E. coli* B/r Thy⁻ strain was synchronized by the sucrose gradient procedure, there was a 20- to 25-min period in which the rate of [$^3$H]thymidine incorporation increased at the beginning of the first cycle only.

In some experiments, the sucrose synchronization procedure slightly lengthened the first synchronous cell cycle relative to the second and third cycles, but the same pattern of [$^3$H]thymidine incorporation was seen in all three cycles.

Figure 2 also shows a graph of the percentage of septated cells at various times throughout the experiment. Septated cells were defined as cells which had a central constriction when viewed in the light microscope. A cell which was seen as septated was counted as one cell in determining the cell number. The number of septated cells was also totaled separately from the cell number determination. The number of septated cells divided by the total number of cells equaled the percentage of septated cells.

**Effect of sucrose gradient synchronization on the cell cycle.** The sucrose gradient method of synchronization might cause some alteration in the normal sequence of events in the cell division cycle (20). To test this possibility, an "inverse synchrony" experiment was per-

formed. An *E. coli* B/r culture growing exponentially in acetate medium was pulsed for 5 min with [$^3$H]thymidine, followed by the addition of a 100-fold excess of nonradioactive thymidine. The uptake of [$^3$H]thymidine was linear with time until the excess of thymidine was added, at which time no more [$^3$H]thymidine was incorporated.

The culture continued to grow exponentially, and two samples were taken every 10 min for 100 min after the initial pulse. The samples were immediately fixed with 2% formaldehyde and placed on ice. One set of samples was used to determine cell numbers during the experiment; cells from the other set of samples were centrifuged, resuspended, and placed on 5 to 30% sucrose gradients. Each sample was centrifuged on a separate sucrose gradient, and the upper layer of small cells, equal to 5% of the total cell population, was removed after centrifugation. These small cells were analyzed for radioactivity. In each sample, the number of [$^3$H]thymidine counts was proportional to the number of these small cells collected. The radioactivity per cell was calculated for each sample with the result shown in Fig. 3.

This experiment shows that the cell cycle is not seriously disrupted by the sucrose gradient technique for synchronization, since the results agree with those of the previous experiment (Fig. 1). The samples removed 10 and 20 min after the [$^3$H]thymidine pulse contained less radioactivity; these cells originated from cells which were mainly 10 to 20 min from dividing and hence were not synthesizing DNA at the time of the pulse. Secondly, a gradual rise in counts through the rest of the time period probably occurred because some of the nonlabeled cells were collected in the intermediate fractions, since controls showed that addition of nonradioactive thymidine stopped incorporation.

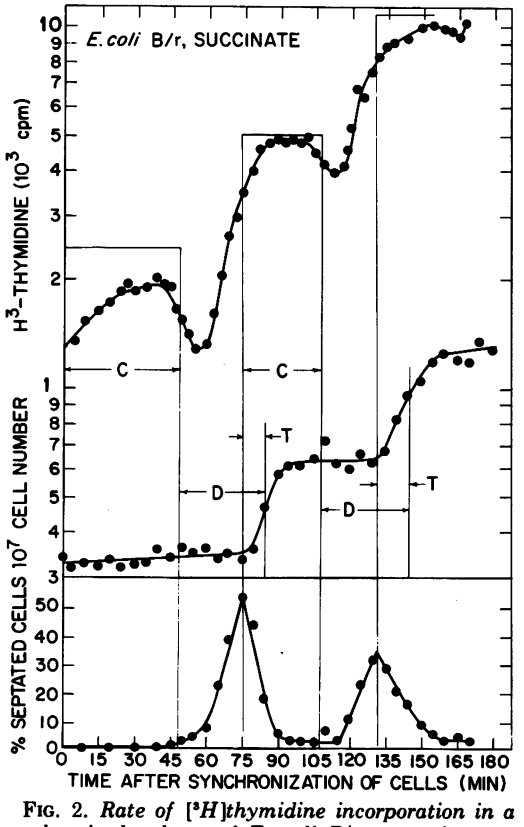


FIG. 2. *Rate of [$^3$H]thymidine incorporation in a synchronized culture of E. coli B/r grown in 0.1% succinate. Upper line: [$^3$H]thymidine pulses; middle line: cell number. Lower line: percentage of septated cells. Septa visible under phase-contrast microscopy were counted.*

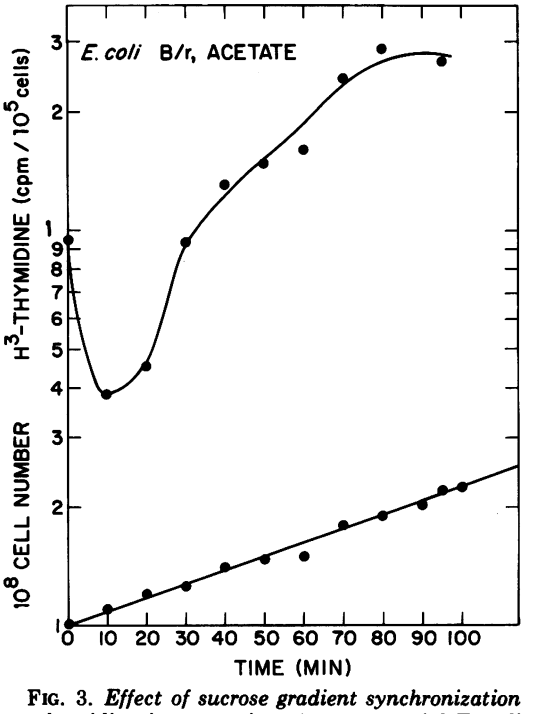


FIG. 3. *Effect of sucrose gradient synchronization on thymidine incorporation. An exponential E. coli B/r culture growing in 0.1% acetate was pulsed with [$^3$H]thymidine for 5 min, followed by the addition of an excess of nonradioactive thymidine. Samples were removed from the exponential culture at 10-min intervals and placed on 5 to 30% sucrose gradients. The smallest cells were removed from the top of the gradients, and the amount of [$^3$H]thymidine per cell was calculated. Upper line: [$^3$H]thymidine counts per min per $10^5$ cells. Lower line: increase in cell number in the exponential culture during the experiment.*

**DNA synthesis in the division cycle of E. coli K-12.** In acetate medium, the *E. coli* strain K-12 3000 had a generation time of 100 to 103 min. The period in which the cell number doubled was equal to 10 to 15 min, which is equivalent to the synchrony obtained by most synchrony methods. The 60- to 65-min period during which DNA synthesis occurred was at the beginning of each cell division cycle, followed by a gap in DNA synthesis during the last one-third of the cell cycle (Fig. 4).

The *E. coli* strain K-12 3000 growing on 0.1% succinate had a generation time of 72 to 74 min. A new cycle of DNA synthesis began prior to cell division, and a period of decreased [$^3$H]thymidine incorporation preceded this new round of DNA synthesis (Fig. 5). Thus, the pattern of DNA synthesis in this strain was similar to the pattern of DNA synthesis exhibited by *E. coli* B/r growing in succinate. However, in *E. coli* K-12 3000 the depth of the gap in DNA synthesis was smaller than in *E. coli* B/r. Presumably, this was caused by a greater amount of asynchrony in the K-12 strain, as compared to *E. coli* B/r, which made a gap in DNA synthesis more difficult to detect. The K-12 strain had a more spherical shape than the B/r strain, which made gradient separation less precise for the K-12 strain.

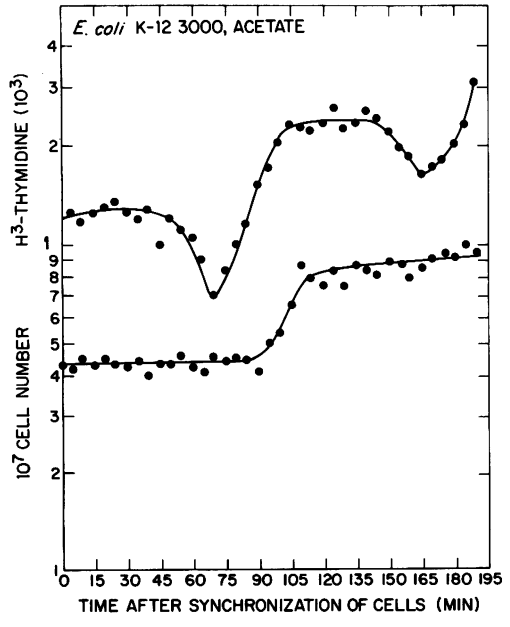


FIG. 4. *Rate of [$^3$H]thymidine incorporation during the cell cycle of E. coli K-12 3000 growing in 0.1% acetate. Upper line: rate of [$^3$H]thymidine incorporation, in counts per minute. Lower line: cell number.*

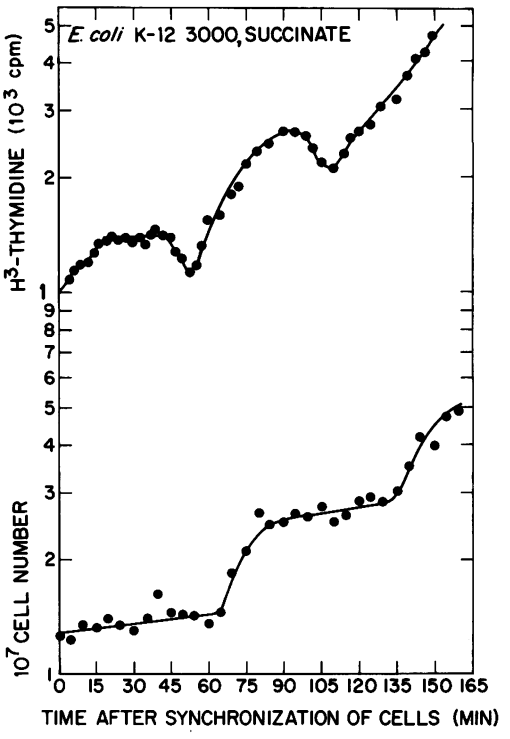


FIG. 5. *Rate of [$^3$H]thymidine incorporation during the cell cycle of E. coli K-12 3000 growing in 0.1% succinate. Upper line: rate of [$^3$H]thymidine incorporation. Lower line: cell number.*

**Division cycles of E. coli 15 and E. coli 15T$^-$.** *E. coli* 15 growing in acetate had a generation time of 62 to 75 min; when *E. coli* 15 was grown in succinate, the length of the first generation after synchronization was 62 min (Fig. 6). In both carbon sources, there was a period late in the cell cycle during which the rate of [$^3$H]thymidine incorporation decreased. The increase in the rate of [$^3$H]thymidine incorporation during the first 15 min after synchronization of the acetate-grown *E. coli* 15 strain was probably caused by the synchronization procedure. In later cycles an increase in the rate of [$^3$H]thymidine incorporation immediately after cell division was never seen.

Thus, *E. coli* 15 was similar to *E. coli* B/r in that it had a period late in the cell cycle devoid of DNA synthesis. However, DNA initiation occurred earlier in the cell cycle in this strain, and the gap in DNA synthesis was slightly longer relative to the length of the period of DNA synthesis.

The *E. coli* strain 15T$^-$ growing in acetate had a generation time of 65 to 70 min when either 10 or 50 $\mu$g of thymine per ml was added to the culture. At a cell age of 0.57 there was a

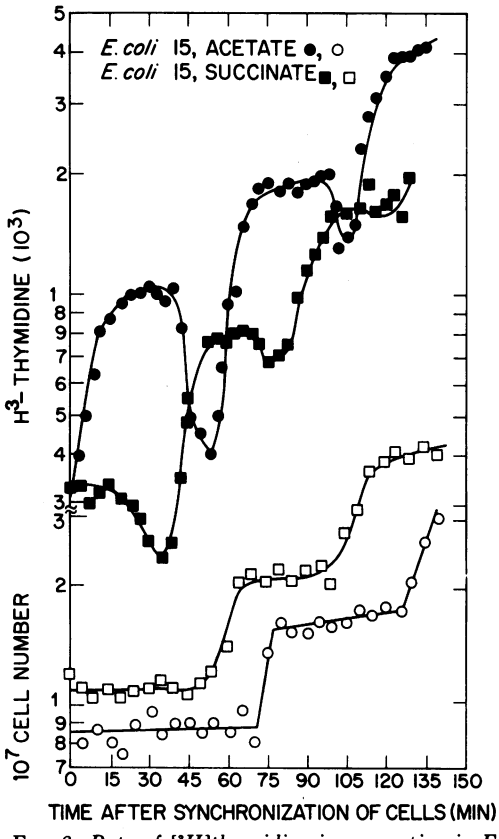


FIG. 6. *Rate of [³H]thymidine incorporation in E. coli 15. Rate of [³H]thymidine incorporation when cells were grown in 0.1% acetate (●) or 0.1% succinate (■), in counts per minute. Cell number when E. coli 15 was grown in acetate (○) or in succinate (□).*

gap in DNA synthesis; this was followed by initiation of a new round of DNA synthesis at a cell age of 0.88 (Fig. 7).

In low concentrations of thymine (0.5 to 1.0 μg of thymine per ml), some cells formed long filaments with no division septa, causing the cell number to increase by less than a factor of two at each synchronous division. No gaps in DNA synthesis were seen when cells were grown in either acetate or aspartate plus low concentrations of thymine, but because of the poor synchrony observed in these cell populations, the absence of a gap may be interpreted as either due to lack of synchrony or due to a longer replication period in each cycle (21).

In succinate, the 15T⁻ strain had a generation time of 76 min, somewhat longer than the 62-min generation time of strain 15 in this medium. The longer generation time may have been due to the thymine requirement of this strain. The length of the C period in this strain was 42 min, and the C period was followed by a gap in DNA synthesis of 24 min. The cell age at initiation was 0.86, in fractions of a generation (Fig. 7). In aspartate (generation time equals 115 to 120 min), the 15T⁻ cells exhibited a small decrease in the rate of [³H]thymidine incorporation late in the cell cycle (Fig. 8). A similar pattern of [³H]thymidine incorporation was observed when 15T⁻ cells were grown in proline with a generation time of 160 to 165 min (Table 1).

Unlike the *E. coli* B/r cells, the rate of [³H]thymidine incorporation was not constant in the first and second cell cycles of 15T⁻ cultures in aspartate. We have evidence that the sucrose synchronization procedure may have damaged the thymidine uptake system transiently, and the increase in rate during the first 40 min reflects recovery of the cells from the sucrose synchronization, as follows.

An exponentially growing culture was subjected to sucrose gradient synchronization, after which the entire cell population was collected from the gradient, placed into fresh medium, and allowed to grow. When acetate-grown *E. coli* B/r cells were used, the rate of [³H]thymidine incorporation in the first 5 to 10 min after

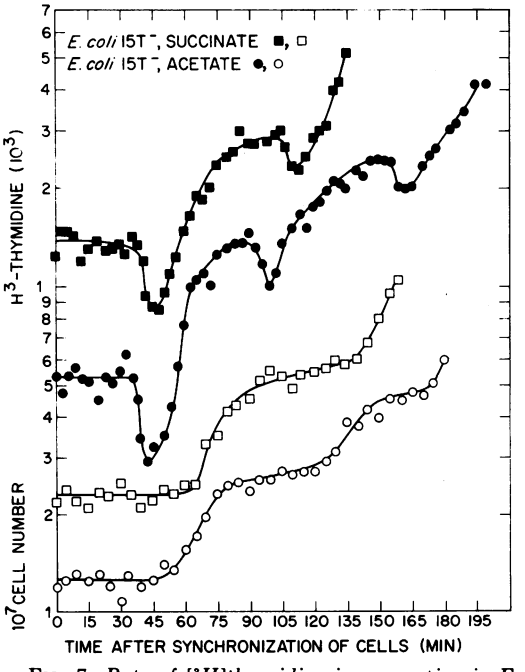


FIG. 7. *Rate of [³H]thymidine incorporation in E. coli 15T⁻. Rate of [³H]thymidine incorporation when cells were grown in 0.1% acetate (●) or 0.1% succinate (■), in counts per minute. Cell number when E. coli 15T⁻ was grown in acetate (○) or succinate (□).*

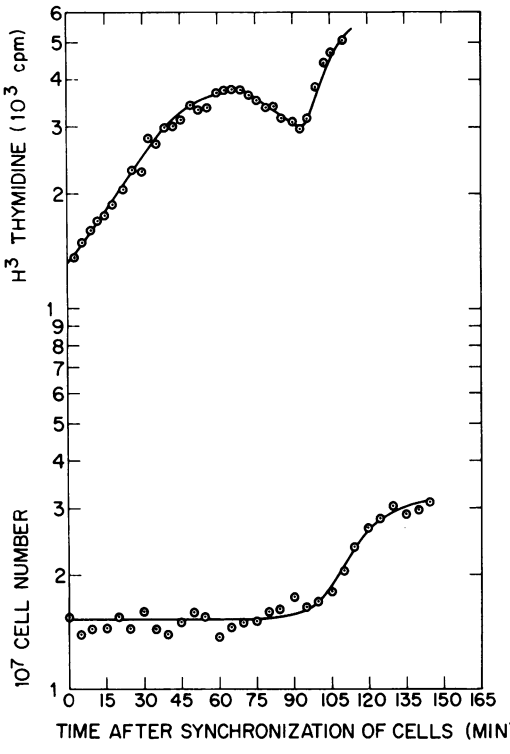


FIG. 8. *Rate of [$^3$H]thymidine incorporation during the cell cycle of E. coli 15T⁻ growing in 0.1% aspartate. Upper line: rate of [$^3$H]thymidine incorporation. Lower line: cell number.*

subjection of the culture to sucrose was slightly lower than the exponential rate of incorporation obtained after the first 10 min. Aspartate-grown *E. coli* 15T⁻ cells incorporated [$^3$H]thymidine at a reduced rate for the first 30 min after subjection of the cells to sucrose, after which they returned to an exponential rate of incorporation.

## DISCUSSION

In all the strains that were studied, gaps in DNA synthesis were only observed late in the cell cycle (Fig. 1 to 8). In addition, the initiation of new rounds of DNA synthesis occurred before the actual physical separation of the two sister cells in all the strains. In fact, the initiation of a new round of DNA synthesis could be correlated with the formation of a cross-wall visible in the light microscope in the strains.

The C period, or period of DNA synthesis, for acetate-grown B/r, K-12, and 15 cells was 60 to 65% of the generation time, whereas in strain 15T⁻ the C period was 57% of the generation time. For the strains growing in succinate, the C period covered 55 to 67% of the cell cycle; the length of time between the end of a round of DNA synthesis and cell division (D period) was 33 to 45% of the cell cycle when cells were grown in succinate, which is longer than the 33% proposed by Cooper and Helmstetter (5) and Pierucci (19).

Some evidence suggests that slowly growing cells might be regulated in a manner different from cells growing with generation times of between 20 and 60 min (7, 9). The correlation of DNA initiation with cross-wall formation may be a means by which slowly growing cells regulate their growth.

By measuring the T period, the period from septation to physical division, we could examine this phase of the cell division cycle more closely. Of course, the measurements of the T period depend on the degree of synchrony in the cell population and on the degree of magnification used when examining the cells. Thus, this calculation of the length of the T period must be considered as a calculation of the minimum length of the T period—better magnification of the cells might show a longer T period. Our results indicated that the T period was approximately 10 to 15 min in length for all cell strains, when succinate, acetate, aspartate, or proline was used as a carbon source. The length of the T period may vary with the length of the cell cycle, however, since Clark (3) reported a T period of 5 to 7 min when he grew *E. coli* B/r/1 in glucose minimal medium with a generation time of 45 min. There is a very short T period when *E. coli* cells are grown at 37 C with a generation time of 21 min (14).

When 15T⁻ cells were grown in aspartate or acetate plus a very low concentration of thymine (0.5 to 1.0 μg/ml), we found that the length of the T period increased to 20 to 25 min, although the generation time did not change. Cells form filaments when DNA synthesis is stopped by nalidixic acid (3) or when Thy⁻ cells are starved for thymine. Thus, a very low thymine concentration in the medium which reduces the rate of DNA synthesis (21) presumably reduces the rate of septum formation and cell separation while not affecting cell mass increase.

The sucrose gradient method for obtaining cell synchrony resulted in a minimum of two and a maximum of five synchronous divisions in the various strains. Gaps in DNA synthesis could not usually be seen after two to three synchronous divisions, however, due to the increasing amount of asynchrony in the cultures.

Although the first cell cycle after collection of the cells from the gradient was longer than the

succeeding cell cycles in some experiments (10), the same pattern of DNA synthesis was seen in the first, second, and third cell cycles. Thus, although there were small effects of the synchronization procedure on the cell cycle, these effects were not pronounced enough to alter the major conclusions made. The DNA synthesis period was always the same fraction of the cell cycle, since the C period also decreased in length during the shorter second cycle.

Synchrony of cell division does not necessarily guarantee synchrony of DNA replication (20); but in these experiments a strong correlation does exist between DNA synthesis cycles and cell division cycles: as the cell number doubles, the rate of [$^3$H]thymidine incorporation also doubles. Better cell division synchrony, that is, a short period of cell number doubling relative to the length of a cell cycle, also corresponded to a better doubling of the rate of [$^3$H]thymidine incorporation. Cells synchronized by the sucrose gradient technique are not synchronized because of exposure to sucrose, but are synchronized on the basis of size, because first, the cells are in sucrose only 10 to 15 min, which is a small fraction of generations times greater than 60 min. In addition, cells growing at slow growth rates (in acetate or succinate) could not be synchronized by growth for several generations in 10% sucrose (20). However, some glucose-grown strains of bacteria can be synchronized by growth in 10% sucrose for several generations, followed by the removal of the sucrose (20). After the removal of sucrose, cells in minimal medium plus glucose swell, and a synchronous division occurs.

## ACKNOWLEDGMENTS

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