REGULATION OF CELL DIVISION IN *ESCHERICHIA COLI*

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ABSTRACT The rate of cell division was measured in cultures of *Escherichia coli* B/r strain after periods of partial or complete inhibition of deoxyribonucleic acid (DNA) synthesis. The rate of DNA synthesis was temporarily decreased by removing thymidine from the growth medium or replacing it with 5-bromouracil. After restoration of DNA synthesis, a temporary period of accelerated cell division was observed. The results were consistent with the idea that chromosome replication begins when an initiator complement of fixed size accumulated in the cell. The increase in the potential for the initiation of new replication points during inhibition of DNA synthesis results in an increase in the rate of cell division after an interval which encompasses the time for the arrival of these replication points to the termini of the chromosomes and the time from this event to division.

INTRODUCTION

In bacteria, chromosome replication and cell division appear to be coordinated such that the frequency of initiation of replication determines the rate of cell division (see Maaløe and Kjeldgaard, 1966). In *E. coli* B/r these two events are linked as follows: (*a*) replication is initiated at a fixed point on the genome (Helmstetter, 1968 *a*; Pato and Glaser, 1968; Donachie and Masters, 1968), (*b*) the replication point traverses the genome in about 40 min in cells growing more rapidly than one doubling per hour, and in about two-thirds of the generation time in cells growing more slowly, and (*c*) cell division occurs about 20 min after the arrival of the replication point at the terminus of the chromosome in cells growing more rapidly than one doubling per hour, and about one-third of the generation time after termination in cells growing more slowly (Cooper and Helmstetter, 1968; Helmstetter, 1968 *b*).

Initiation of chromosome replication requires protein synthesis (Maaløe and Hanawalt, 1961; Lark et al., 1963) and depends on the achievement of a critical cellular mass/DNA ratio (Hanawalt et al., 1961). Recently, it has been suggested that chromosome replication begins when a fixed amount of "initiator" has accumulated (Helmstetter, 1968 *b*; Donachie and Masters, 1968). When this quantity has accumulated, new rounds of replication begin regardless of the position of

other replication points on the chromosome (Maaløe and Rasmussen, 1963). Thus, the rate of cell division in a given growth medium is probably determined by the time for accumulation of the initiator in that medium. It follows that if DNA synthesis were selectively inhibited in a culture, initiator would continue to accumulate, resulting in an increase in the potential for the inception of new replication points. After restoration of DNA synthesis, a period of accelerated cell division would be expected. In the present study, the rate of cell division was measured in cultures of *E. coli* B/r T^- in which the rate of DNA synthesis per replication point was temporarily reduced by removing thymidine from the medium or replacing it with 5-bromouracil. The rate of cell division following these treatments was consistent with the hypothesis of an accumulation-like behavior for the initiator.

MATERIALS AND METHODS

Bacteria and Growth Conditions

The organism used was an auxotroph of *E. coli* B/r T^- His^- (obtained from Dr. J. D. Friesen). The minimal salts medium contained 2 g NH_4Cl, 6 g Na_2HPO_4, 3 g KH_2PO_4, 3 g NaCl, and 0.25 g $MgSO_4$ in 1 liter of distilled, demineralized water. Glucose (1 mg/ml), thymidine (15 μg/ml), and histidine (50 μg/ml) were added to this medium.

The experiments were performed on portions of 100 ml cultures which had incubated for approximately 17 hr at 37°C with shaking and were at concentrations of approximately 1×10^8 cells/ml in exponential growth. Medium transfers were done by filtering parts of this culture onto membrane filters, washing with 100 ml of prewarmed medium without thymidine (−T–medium) or containing 5-bromouracil (8 μg/ml if not otherwise indicated) and no thymidine (BU–medium), and resuspending in 100 ml of −T–medium or Bu–medium, respectively. Normal conditions of growth were restored by adding 15 μg/ml of thymidine directly to the thymidine starved, and 5-bromouracil–treated cultures. Control cultures were subjected to similar manipulations, but were washed and resuspended in medium containing thymidine (+T–medium). If not otherwise indicated, the amount of cells to be transferred was chosen so that the concentration of the cells at the time of selection of newborn cells was $0.5–1.0 \times 10^8$ cells/ml. Newborn cells were selected from the culture by using the membrane-elution technique of Helmstetter and Cummings (1964). The 100 ml cultures were poured onto a 0.22 μ GS Millipore filter (Millipore Filter Corp., Bedford, Mass.) and attached to the membrane by suction. After washing, the membrane was inverted and eluted with prewarmed media at a rate of 3–4 ml/min. The experimental details have been described previously (Helmstetter, 1967).

Density Gradient Sedimentation Analysis

A 100 ml culture containing 10^8 cells/ml growing exponentially in minimal salts medium containing thymidine was transferred by filtration into 150 ml of medium containing 4 μg/ml 5-bromouracil and 0.1 μc/ml 5-bromouracil-2-^{14}C (International Chemical and Nuclear Corp., City of Industry, Calif.). 10 ml aliquots of the culture were withdrawn every 20 min for 200 min, filtered onto membranes, and resuspended into cold 0.1 M Tris buffer (pH = 8.0). The cells were harvested by centrifugation at 1800 × *g* for 30 min at 4°C in a PR-2 International Refrigerated Centrifuge (International Equipment Co., Needham Heights, Mass.). The resulting pellets were frozen for analysis at a later time.

0.5 ml of 0.01 M Tris buffer (pH = 8) containing 0.4% ethylenediamine-tetraacetic acid (EDTA) and 1% sodium lauryl sulfate (duponol), and 0.2 ml of 1 mg/ml lysosyme were added to the pellets and the cells were disrupted by rapidly freezing in dry ice–acetone mixture followed by thawing at 37°C. After 30 min of incubation at 37°C, 0.2 ml of 1 mg/ml pronase was added, and the mixture was incubated at 37°C for another 30 min. Then, 0.2 ml of the suspension and 4.8 ml of a CsCl solution of final average density of 1.76 g/cm^3 and pH = 8.0 were poured into a cellulose nitrate centrifuge tube and overlaid with mineral oil. Centrifugation was performed in a Spinco Model 50w Swinging Bucket Rotor (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) at 10°C, 33,000 rpm for 72 hr. Fractions of 15 drops (approx 0.15 ml) were collected on fiber glass filters (Whatman). After drying, the radioactivity on the filters was measured with a Nuclear-Chicago (Nuclear-Chicago Corporation, Des Plains, Ill.) liquid scintillation counter.

Cell Concentration, Turbidity, and Viability Counts

The concentration of cells was determined with a Coulter Counter Model F (Coulter Electronics Inc., Hialeah, Fla.). Viable cell counts were made in triplicate by the pour plate method using nutrient agar plates. Absorbancies at 450 mμ were determined in 1 cm light-path cuvettes with a Beckman Model DB Spectrophotometer.

Thymidine-^{14}C Incorporation

1 μc of thymidine-^{14}C (2 c/mM, New England Nuclear Corp., Boston, Mass.) and 40 μg of unlabeled thymidine were added to 10 ml samples of cells for a 40 min incorporation period. Cold trichloroacetic acid (TCA) at 10% final concentration was added at the end of the incorporation, and after 30 min in an ice bath, samples were filtered onto 0.45 μ type GS Millipore filters. All TCA solutions contained 100 μg of thymidine per ml. The radioactivity of the filters was measured as described above.

MODEL

The theoretical rate of cell division following temporary, selective inhibition of DNA synthesis can be determined if initiation of chromosome replication depends upon the accumulation of a specific amount of non-DNA material. The theoretical calculations are based on the following assumptions concerning initiation and cell division:

(*a*) An "initiator" complement of fixed size must be synthesized to induce replication of a chromosome (at a given temperature). This quantity of "initiator" is the same in all physiological states (Helmstetter, 1968 *b*; Donachie and Masters, 1968);

(*b*) rounds of chromosome replication start at a fixed point on the genome called the replication origin (Lark et al., 1963; Abe and Tomizawa, 1967; Helmstetter, 1968 *a*; Wolf et al., 1968);

(*c*) rounds of replication start simultaneously at all chromosome origins in the cell;

(*d*) each time a new round of replication is initiated, a division follows in $(C + D)$ min where C is the time for a replication point to traverse the chromosome (a round

of replication) and D is the time between the end of a round of replication and cell division (Cooper and Helmstetter, 1968); and

(*e*) the time interval (D) between the completion of a round of chromosome replication and cell division is not altered by selective inhibition of DNA replication (Helmstetter and Pierucci, 1968).

For simplicity, we also have considered that a *new* complement of initiator must be synthesized to start a new round of replication, and that the rate of initiator synthesis per origin is constant in a given medium. Consequently, the rate of synthesis per cell should depend upon the number of origins.

If the rate of DNA synthesis per replication point were reduced in an exponentially growing culture, the rate of cell division would remain unchanged for D min since all cells which had completed a round of chromosome replication at the start of the treatment would divide in the normal sequence (Helmstetter and Pierucci, 1968; Clark, 1968). After this time, division would stop if DNA synthesis were stopped and it would continue at a reduced rate if the rate at which replication points arrived at the chromosome termini were reduced. During the treatment periods, the capacity for initiation would continue to accumulate at the pretreatment rate. Upon restoration of the normal rate of DNA synthesis, no change in the rate of cell division should occur for D min and then the pattern of cell division should reflect the prior accumulation of initiator, i.e., the presence of multiple replication points per chromosome.

RESULTS

Kinetics of Chromosome Replication and Cell Growth During Limited DNA Synthesis

As preliminaries to measurement of the rate of cell division following temporary alteration of DNA synthesis in *E. coli* B/r T^-, the kinetics of DNA synthesis in BU-medium, and the effects of thymidine starvation and incubation in BU-medium on cell growth were determined.

The kinetics of chromosome replication in *E. coli* B/r T^- grown in BU-medium was found by following the appearance of bromouracil-^{14}C in material which banded at 1.80 g/cm^3 during CsCl density gradient centrifugation (heavy DNA). As shown in Fig. 1, radioactivity began to appear in heavy DNA after about 120–140 min of incubation in BU-medium. Thus, the apparent minimal time for a round of replication in BU-medium was about 120 min. The time required for a round of replication in +T-medium was determined as described by Helmstetter (1967) and found to be about 40 min. The increase in the apparent time for a round of DNA replication from 40–120 min in BU-medium could have been a consequence of either a reduction in the rate of DNA synthesis at individual replication points or a normal rate of synthesis followed by a period of no synthesis at the time at which 5-bromouracil should substitute thymine into the two complementary strands of DNA.

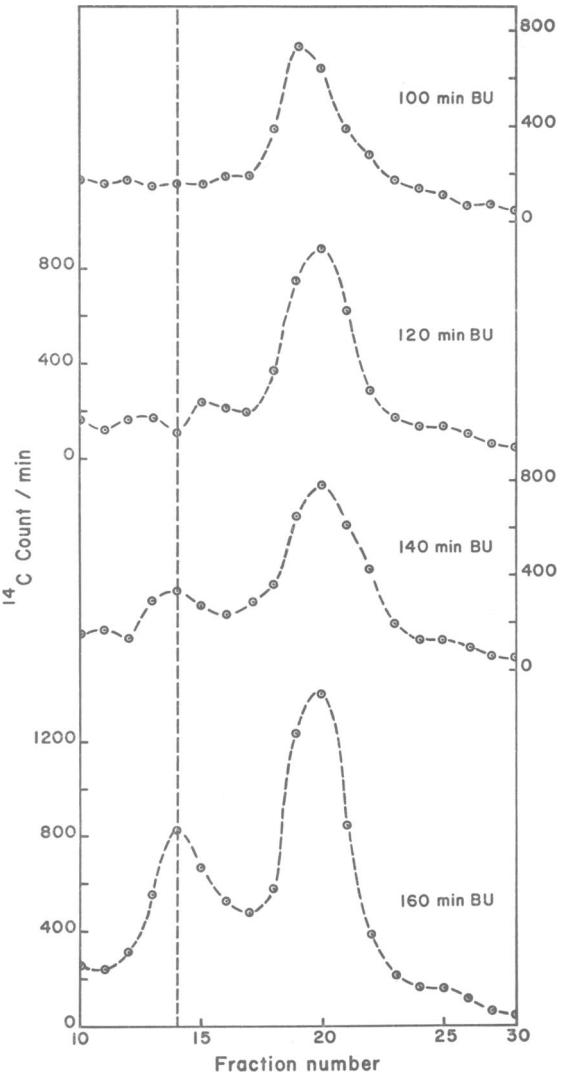


FIGURE 1 Radioactivity profile of density gradient sedimentation patterns of lysates of cells growing in glucose minimal medium. Bromouracil-5-^{14}C was substituted for thymidine at 0.1 μc/ml for 100, 120, 140, and 160 min as indicated. The vertical line indicates the approximate position of DNA containing 5-bromouracil in place of thymine in both strands.

When cultures were incubated for different times with BU-medium and then exposed to a 15 min pulse of thymidine-^{3}H, the tritium label was found in hybrid DNA after about 60 min of 5-BU incubation. The early appearance of radioactivity in the hybrid DNA was consistent with, but not conclusive of, the introduction of extra replication points in the slowly replicating genome. This point was further

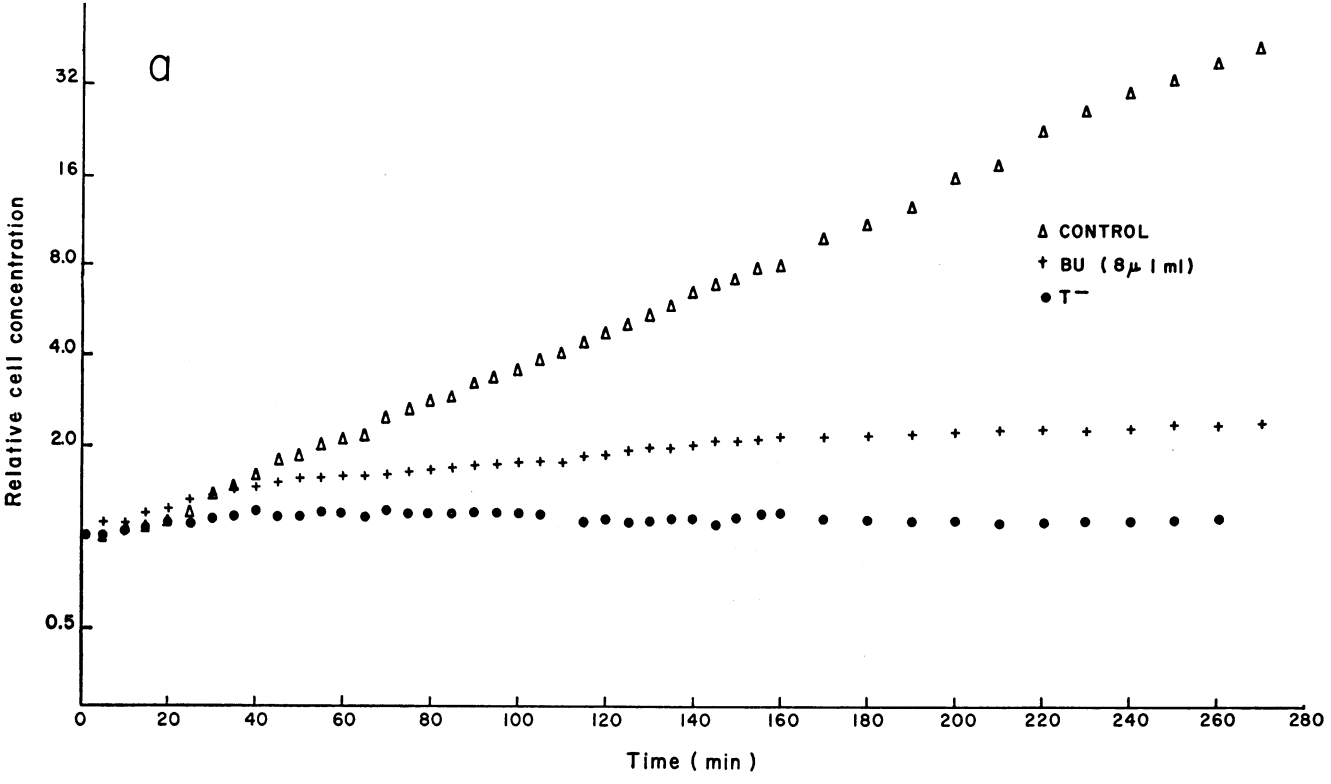


FIGURE 2 Growth characteristics of cell cultures in glucose minimal medium: (*a*) cell concentration, (*b*) turbidity, (*c*) colony-forming ability. 20 ml of approximately 1×10^8 cells/ml culture growing exponentially were transferred, after washing, into 100 ml of fresh medium containing 15 μg/ml thymidine (△), 8 μg/ml 5-bromouracil (+), and no thymidine (•). After transfer, the concentration of cells was approximately 3×10^7 cells/ml in all cultures.

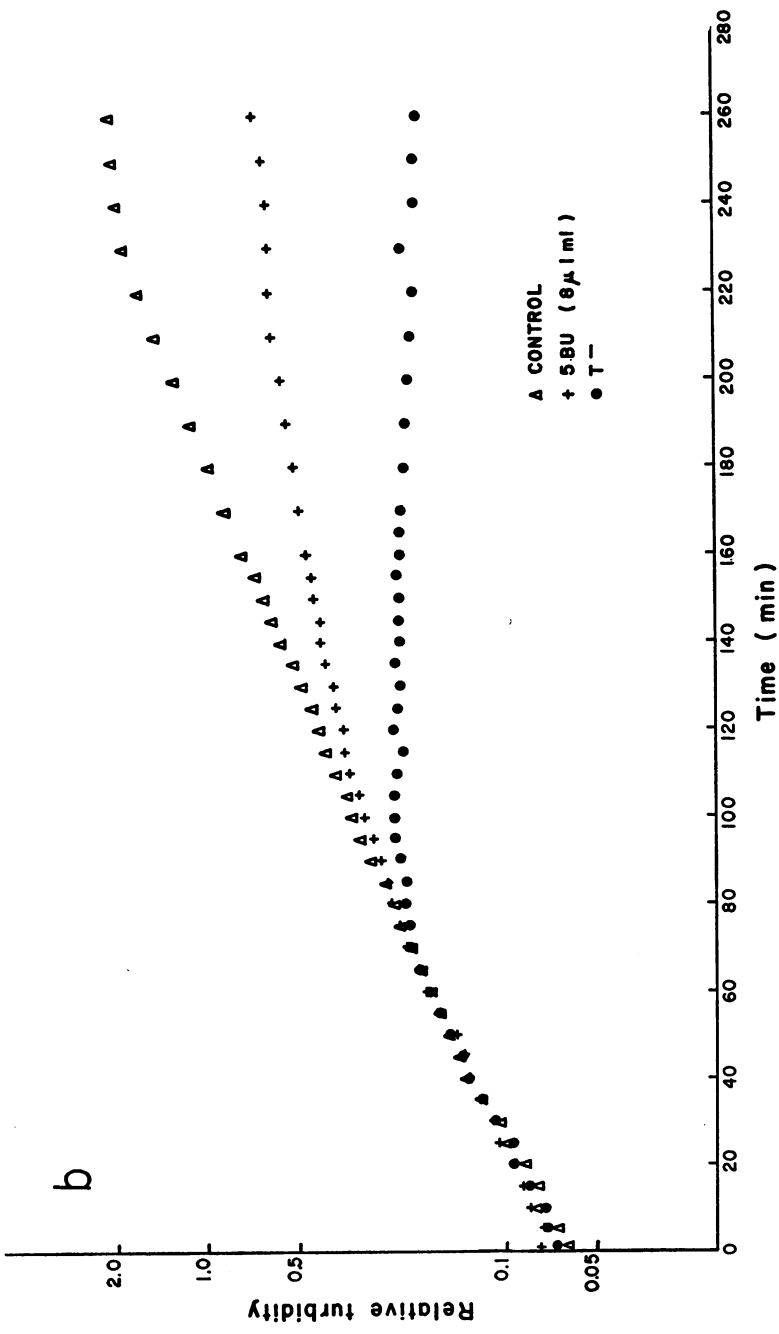

b
Relative turbidity
2.0
1.0
0.5
0.1
0.05
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
Time (min)
Δ CONTROL
+ 5-BU (8μl/ml)
● T⁻

FIGURE 2 *b*

tested by determining the amount of thymidine-^{14}C incorporated by cells which were grown in BU-medium and then incubated with the label in +T-medium (results reported in a later section). It was concluded that the potential for initiation of new rounds of chromosome replication increased continuously during the first 60 min of incubation in BU-medium. Considering all of these observations, a value of 120 min was chosen for *C* in BU-medium for the calculations which follow.

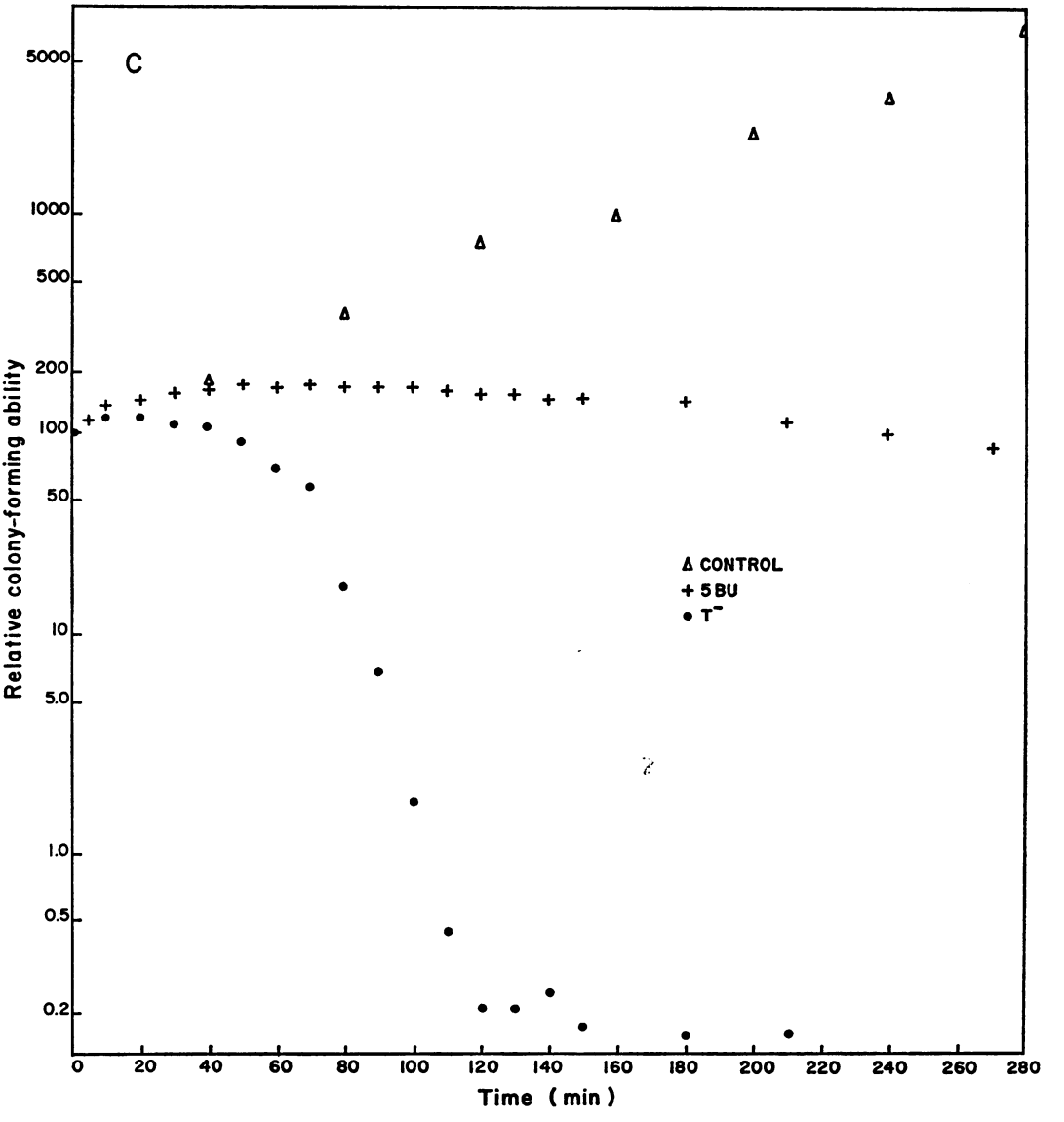


FIGURE 2*c*

The effect on cell growth of thymidine starvation and incubation in BU-medium is shown in Fig. 2. During the treatments, the concentration of cells increased for a short time at a rate similar to the untreated culture (Fig. 2 *a*) and then no further increase was observed in the thymidine-starved culture, whereas the concentration of cells continued to increase slowly in BU-medium. Filtration and resuspension of the cells in prewarmed medium altered the rate of cell division of control cultures, but the rate returned to normal after 25 min.

The turbidity of the treated cultures increased along with the control for the first 60–70 min after removal of thymidine, and for 100–120 min in BU-medium (Fig. 2 *b*). After these times, the turbidity did not increase in thymidine-starved cultures, whereas it continued to increase slowly in cultures growing in BU-medium.

Thymidine starvation did not affect the colony-forming ability of the cells during the first 40–60 min, but after this time viability decreased abruptly (Fig. 2 *c*). Similarly, the viability of BU-grown cells was not impaired for the first 100 min, but it then decreased; although about 50% of the cells were still viable after 220 min in BU-medium. The growth characteristics were independent of the concentration of 5-bromouracil between 2 and 30 μg/ml.

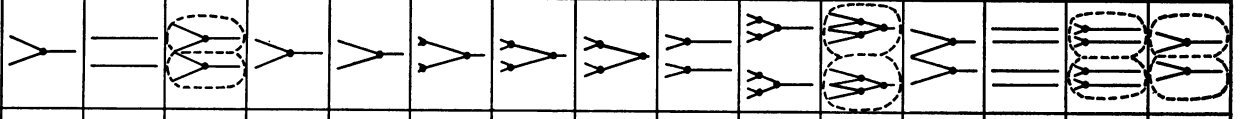
Predicted Effects of Decreasing the Rate of DNA Synthesis in a Population

The values for C used for the theoretical calculations presented in the following sections were based on the idea that growth in +T-medium corresponds to $C = 40$ min, in −T-medium to $C = \infty$, and in BU-medium to $C = 120$ min.

The series of events through which a newborn cell growing in glucose medium would pass if it were shifted from medium in which $C = 40$ min into medium in which $C = 120$ min for 60 min and then back to $C = 40$ min is described in Table I. (Throughout this section it is assumed that $D = 20$ min in all cases [Cooper and Helmstetter, 1968].) During balanced growth in medium with $C = 40$ and $D = 20$ min, cells of age 0 contain one half-replicated chromosome, cells of age 0.5 contain two completed chromosomes, and cells of age 1.0 contain two half-replicated chromosomes (Forro and Wertheimer, 1960; Koch, 1966; Clark and Maaløe, 1967; Helmstetter, 1967; Cooper and Helmstetter, 1968). It has been assumed that the rate of initiator synthesis is unaffected by the shift in medium, i.e., that a unit of initiator accumulates per origin every 40 min. A unit of initiator is defined as the amount required to induce a replication point. During 10 min in the medium with $C = 120$ min, a replication point traverses only $\frac{1}{12}$ of the genome but the amount of initiator which accumulates (0.25 units per origin) is the same as before the shift. When the amount of initiator reaches one complete unit per origin, new replication points are initiated at all origins and these new replication points proceed along $\frac{1}{120}$ of the genome per minute. Upon returning to $C = 40$ min after 60 min of $C = 120$ min (at 100 min in Table I), the replication points again progress along $\frac{3}{12}$ of the genome in each 10 min interval. Since cells divide 20 min after completion of

TABLE I

EFFECT OF 5-BROMOURACIL ON GROWTH CHARACTERISTICS

Treatment time (min) / Cell Characteristics	C = 40'			C = 120'						C = 40'					
	0.0	20.0	40.0 ↓	50.0	60.0	70.0	80.0	90.0	100.0 ↓	106.7	120.0	126.7	140.0	146.7	160.0
Chromosome Configuration															
Fraction of Chromosome Replicated	6/12	12/12	6/12	7/12	8/12	1/12 9/12	2/12 10/12	3/12 11/12	4/12 12/12	2/12 6/12	6/12 10/12	8/12 12/12	12/12	2/12	6/12
Number of Replication Points per Cell	1	1→2	1	1	1→3	3	3	3	3→2→6	6	3	3→2	2→4	2	1
Amount of Initiator Accumulated per Cell	1.00	2.00	1.00	1.50	2.00	1.00	2.00	3.00	4.00	1.33	2.00	2.66	4.00	0.67	1.00
Amount of Initiator Accumulated per Origin	0.50	1.00	0.50	0.75	1.00	0.25	0.50	0.75	1.00	0.17	0.50	0.67	1.00	0.17	0.50

a round of chromosome replication in both media, no cell divisions are observed during the 60 min of $C = 120$ min. However, between 20 and 60 min after restoration of $C = 40$ min, the cells divide three times so that the concentration of cells increase eightfold in 40 min. After this time, the cells divide every 40 min.

Experimental Effects of Decreasing the Rate of DNA Synthesis in a Population

A temporary reduction in the rate of DNA synthesis per replication point would be expected to cause (*a*) an increase in the number of replication points per chromosome and (*b*) a period of accelerated division after restoration of DNA synthesis.

(*a*) *Number of Replication Points Per Chromosome* The appearance of extra replication points can be determined by measuring the rate of DNA synthesis in cells having known chromosome and initiator contents. With *E. coli* B/r this can be done by binding the culture to a membrane filter and collecting the newborn

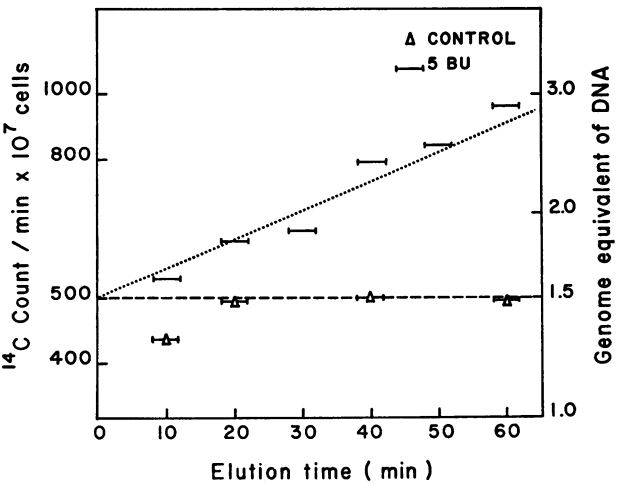


FIGURE 3 Incorporation of thymidine-^{14}C into cells eluted from membrane-bound cultures. 100-ml cultures containing 1.0×10^8 cells/ml growing in glucose minimal medium were bound to membranes, washed, and eluted at a rate of 3 ml/min with medium containing 4 μg/ml of 5-bromouracil. 4 min samples were collected at 10, 20, 30, 40, 50, and 60 min after the start of elution and were poured into culture flasks containing 1 μc of thymidine-^{14}C and 40 μg of unlabeled thymidine. After 40 min of incubation at 37°C with shaking, the incorporation of the radioactive label was stopped by adding cold trichloroacetic acid and excess unlabeled thymidine. Control cultures were eluted with 4 μg/ml thymidine and treated in a similar way. The bar indicates the length of the collection time. The straight lines (dotted, $C = 120$; dashed, $C = 40$) represent the potential for DNA replication calculated assuming the accumulation of initiator. The $C = 120$ min line was determined by calculating (1) the time of division of individual cells in an exponentially growing culture after the shift from $C = 40$ to $C = 120$, (2) the chromosome configurations and the amount of initiator at division, and (3) the number of genome equivalents of DNA replicated in a 40 min period following restoration of $C = 40$ min. The $C = 40$ min curve was determined by calculating the number of genome equivalents replicated in a 40 min period by newborn cells with $C = 40$ min.

cells which elute from the membrane. If a culture growing in a medium in which $C = 40$ min is bound to a membrane and eluted with medium in which $C = 120$ min, the newborn cells would contain, as elution progresses, increasing amounts of initiator or extra replication points on the genome. The potential for DNA replication in newborn cells following a reduction in the rate of DNA synthesis was found by collecting samples of the effluent from membrane-bound cells eluted with BU-medium ($C = 120$ min) and measuring the amount of thymidine-^{14}C incorporated during 40 min of subsequent growth in +T-medium ($C = 40$ min). The experimental results, shown in Fig. 3, are as predicted by the model (see Appendix *A* for detailed calculations).

(*b*) *Cell Division* The manner in which an exponentially growing culture would respond to a shift from $C = 40$ to $C = \infty$ or $C = 120$ min is shown in Fig. 4. The top line shows the concentration of cells in an exponentially growing culture ($C = 40$ min) vs. incubation time. The middle curve shows the concentration of

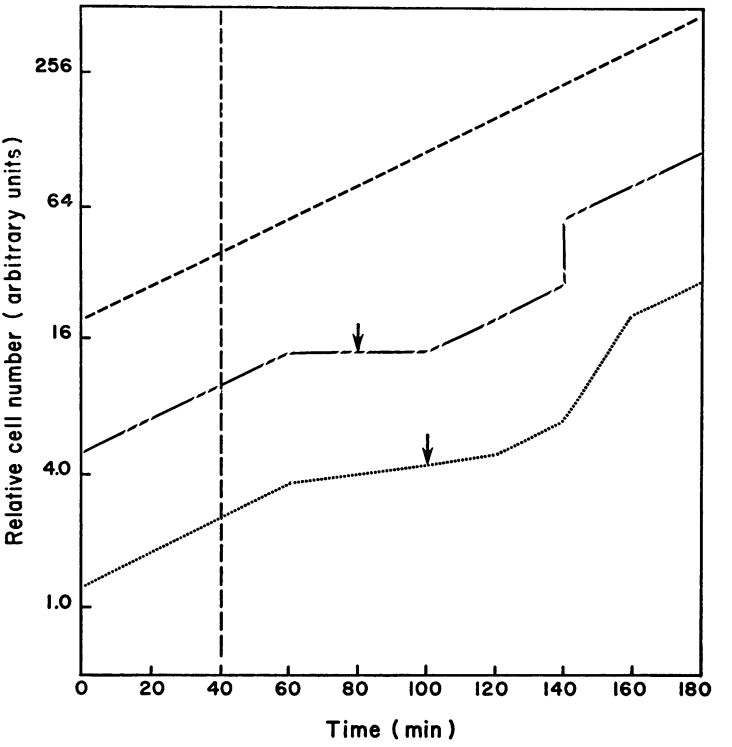


FIGURE 4 Theoretical pattern of cell division in an exponential-phase culture in which DNA replication is selectively inhibited and then restored. The illustrations were constructed by setting the time for synthesis of an initiator complex per origin equal to 40 min, $D = 20$ min and $C = 40, \infty$, or 120 min during unrestricted replication (top line), complete inhibition (middle curve), and partial inhibition (bottom curve) of chromosome replication, respectively. The vertical line indicates the onset of the inhibition and the arrows indicate the restoration of unrestricted replication.

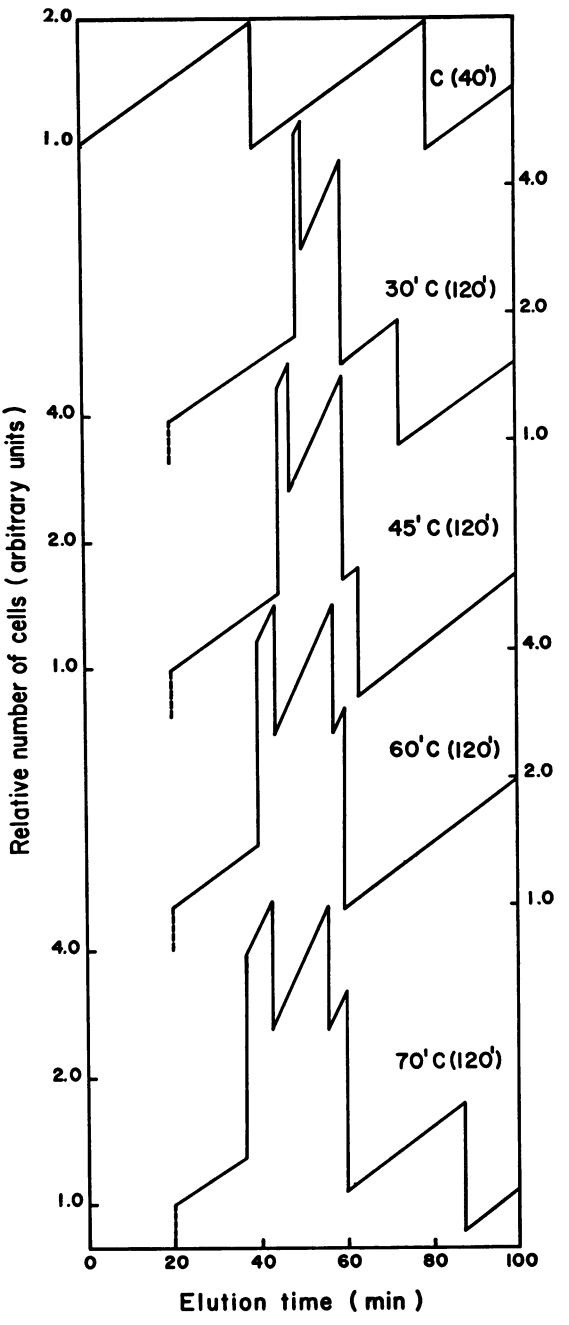


FIGURE 5 *a* Theoretical number of cells in the effluent from membrane-bound populations in which partial inhibition (C = 120 min) of chromosome replication was induced for 30, 45, 60, and 70 min. Unrestricted replication (C = 40 min) was restored at the time the cells were bound to the membrane. The elution pattern was constructed by (1) considering the age distribution of an exponentially growing cell population, (2) extending the calculations in Table I to cells of different ages and, (3) by determining the rate of cell division in cultures which were continuously deprived of half of the newborn cells. The patterns can also be constructed by differentiation of integral curves such as shown in Fig. 4 and considering that one of the two daughter cells eluted from the membrane at division.

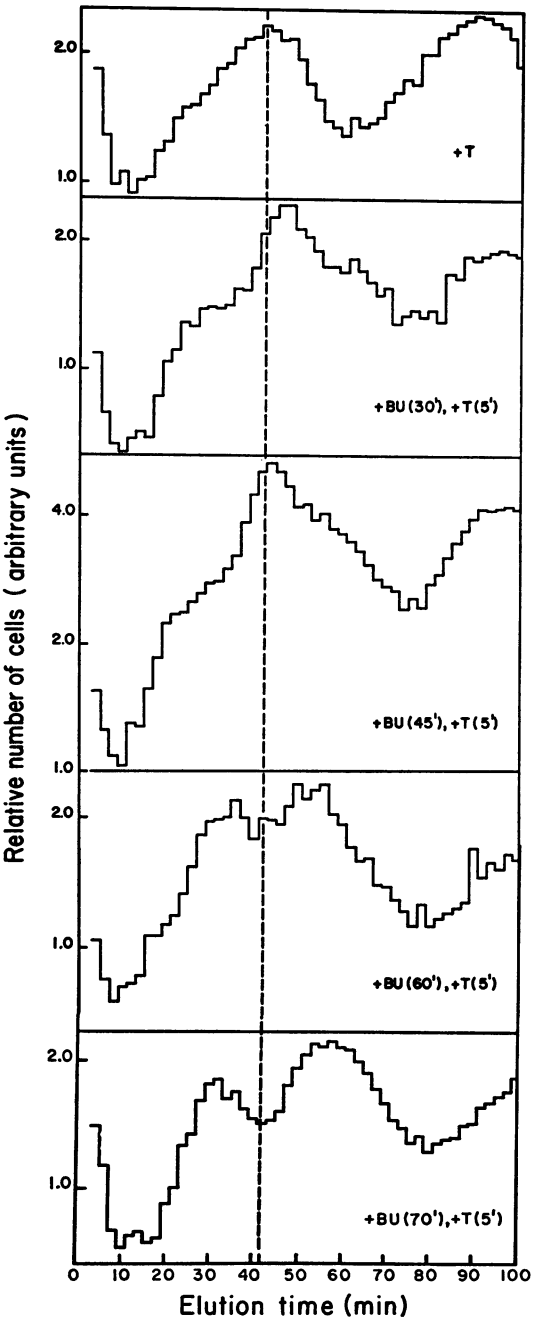


FIGURE 5 *b* Elution of cells from membrane-bound cultures subjected to 5-bromouracil incubation for various times. 100 ml cultures containing 0.5 to 1.0 × 10^8 cells/ml growing in glucose minimal medium were bound to membranes after growth in medium containing 8 μg/ml 5-bromouracil in place of thymidine for 30, 45, 60, and 70 min. 5 min prior to the binding, 15 μg/ml of thymidine was added to the cultures. The cells were washed and eluted with medium containing 15 μg/ml of thymidine. In each experiment, the elution rate and the sampling interval were 4 ml/min and 2 min, respectively. The upper curve shows the elution pattern from an exponential phase culture growing in thymidine. The dotted vertical line indicates the position of the maximal concentration of cells eluted from this culture.

cells in an exponentially growing culture which is shifted to $C = \infty$ at 40 min. The rate of cell division remains unchanged during the first D min (from 40 to 60 min) after the shift. The cells which divide during the D interval had already completed a round of chromosome replication at the time of the shift. Further division is prevented because replication points do not arrive at the terminus of the chromosome while $C = \infty$. Following restoration of $C = 40$ min at 80 min, the preexisting repli-

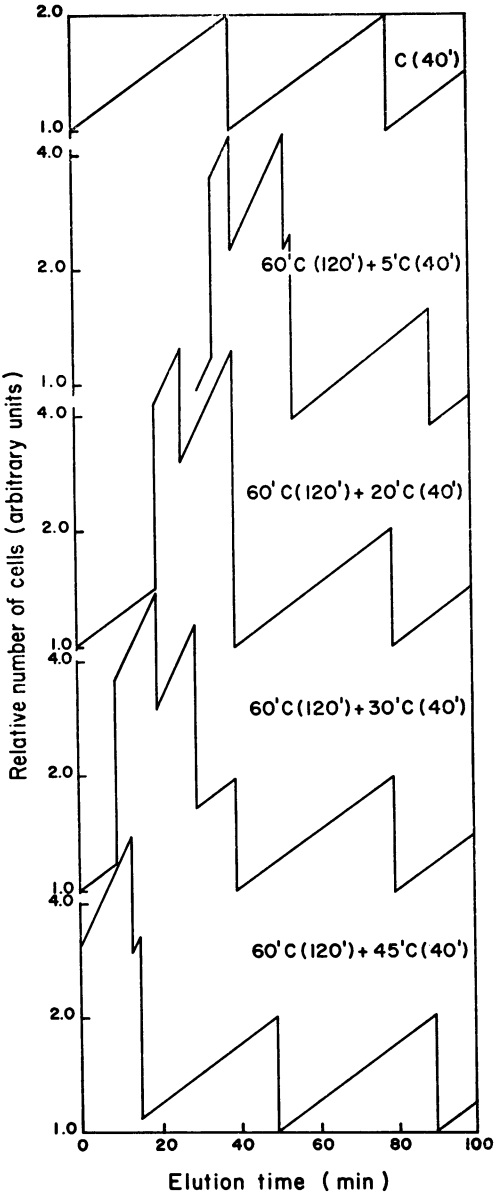


FIGURE 6 *a* Theoretical number of cells in the effluent from membrane-bound populations in which partial inhibition ($C = 120$ min) of chromosome replication was induced for 60 min. Unrestricted replication ($C = 40$ min) was restored 5, 20, 30, and 45 min prior to filtration onto the membranes. Details of the calculations are given in Fig. 5 *a*.

cation points begin arriving at the terminus, and division resumes D min later (at 100 min) at the normal rate. However, during the 40 min period of $C = \infty$, one unit of initiator was synthesized per origin, and when $C = 40$ min is restored extra replication points are introduced at the origin of all chromosomes. All of these

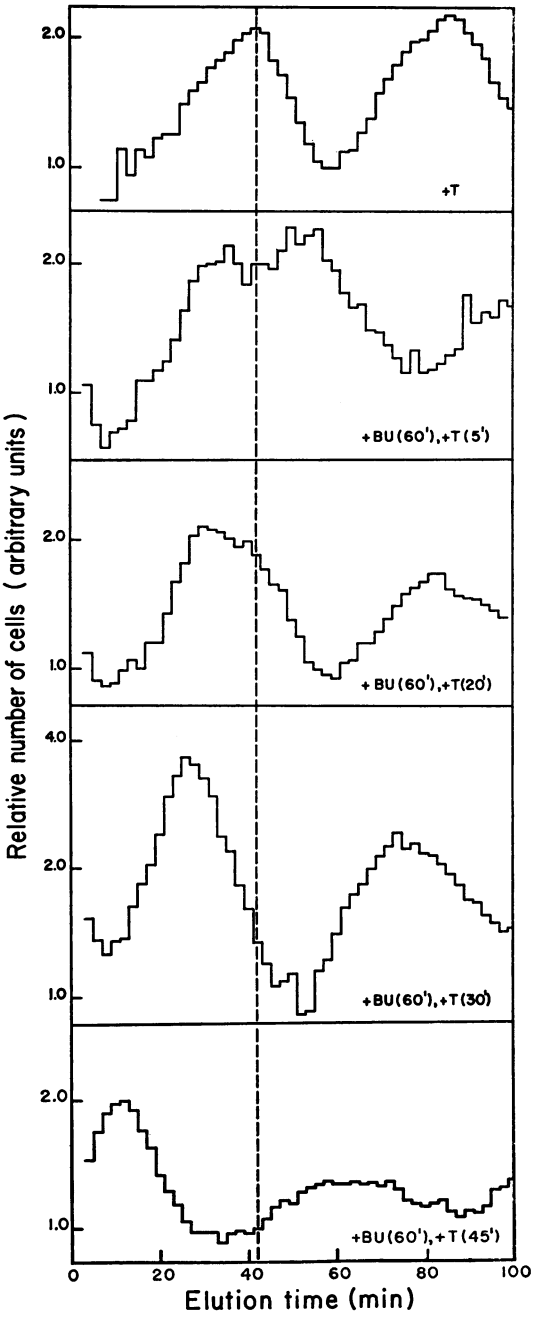


FIGURE 6 *b* Elution of cells from membrane-bound cultures grown in 5-bromouracil medium. After the cultures (100 ml of about 1×10^8 cells/ml) had incubated in BU-medium for 60 min, thymidine was added at 15 μg/ml for 5, 20, 30, and 45 min prior to attachment to the membrane.

replication points arrive at the termini of the chromosomes 40 min later and a synchronous division is expected after D min (at 140 min).

The bottom curve shows the concentration of cells in an exponentially growing culture which is shifted to $C = 120$ min at 40 min. The pattern of cell division was determined by extending the calculations of Table I to groups of cells of different ages, and by accounting for the age distribution of the initial population. The cell concentration increases at the normal rate during the first D min after the shift as in the middle curve. The reduced rate of cell division after this time corresponds to the decreased frequency of arrival of replication points at the termini when $C = 120$ min. The rate of cell division remains unchanged for D min after restoration of $C = 40$ min at 100 min. After D min, the rate of cell division assumes the value characteristic of growth with $C = 40$ min corresponding to the normal frequency of arrival of replication points at the termini. However, during the 60 min period of $C = 120$ min, initiator synthesis continued and extra replication points were inserted on the chromosome when a unit of initiator had accumulated per origin. This results in a period of accelerated division 40–60 min after restoration of $C = 40$ min (at 140–160 min on the figure).

Experimentally, the kinetics of cell division in a culture can be most accurately determined by measuring the *rate* of cell division directly rather than the total number of cells in the population (Helmstetter, 1967). This can be done by measuring the concentration of newborn cells which elute from membrane-bound cultures. Since only one of the two daughter cells that originate at division is eluted, this measurement yields the rate of cell division in a culture continuously deprived of half of its newborn cells. The theoretical elution patterns of cells which were grown in medium with $C = 120$ min for varying periods, bound to membranes, and then eluted with medium in which $C = 40$ min, are shown in Fig. 5 *a*. The curves were obtained by differentiating curves such as shown in Fig. 4, and by considering that one of the two daughter cells eluted from the membrane at division. (See Appendix *B* for detailed calculations.)

Figure 5 *b* shows the concentration of cells in the effluent from membrane-bound cultures which had been grown for different times in BU-medium, bound to membranes, and eluted with +T-medium. During the first 20 min of elution, removal of weakly attached cells masks the low division rate which is anticipated theoretically. After this time, the experimental results were essentially in agreement with the theoretical curves (Fig. 5 *a*) as regards the position of the maxima of cell division. Although no fine structure was resolved, two major peaks appeared in the experimental curves following treatments in which the right side of the peak should have become more prominent theoretically.

Figs. 6 *a* and *b* show the theoretical and experimental elution pattern of cultures grown for 60 min in BU-medium ($C = 120$ min) and restored to +T-medium ($C = 40$ min) for different lengths of time prior to attachment to the membranes. Again, the theoretical and experimental curves are in fundamental agreement.

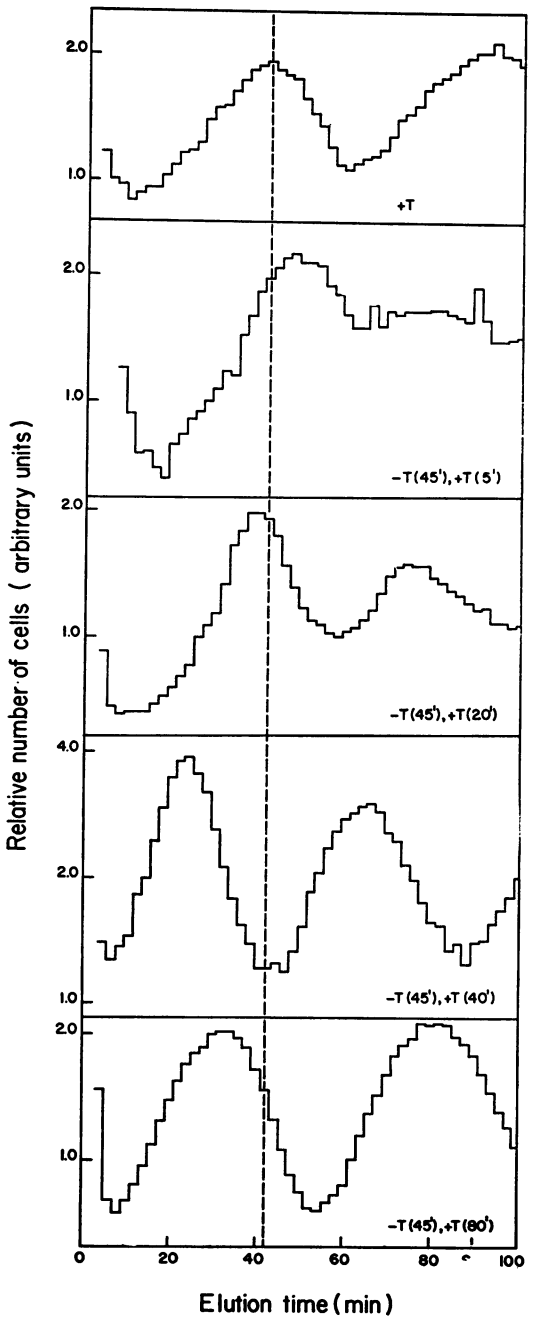


FIGURE 7 Elution of cells from membrane-bound cultures subjected to thymidine starvation. After 45 min of thymidine starvation, thymidine was added to cultures at 15 μg/ml for 5, 20, 40, and 80 min prior to attachment to the membranes.

Fig. 7 shows the elution of cells which had been starved of thymidine for 45 min and then restored to +T-medium for 5, 20, 40, and 80 min prior to attachment of the membranes. In this case, a synchronous division was expected $C + D = 60$ min after restoration of thymidine to the starved cultures. Thus, the first maximum of cell division was expected at 55, 40, 20, and 40 min which is in agreement with the experimental findings of maxima at 50, 40, 25, and 35 min. When +T-medium was restored for 80 min prior to elution, the maximum was expected at 40 min since the synchronous division would occur prior to the attachment to the membrane and the elution pattern of the culture should be back to normal.

DISCUSSION

The general agreement between the experimental and predicted rates of cell division and DNA replication in cultures of *E. coli* B/r subjected to temporary inhibition of DNA synthesis supports the suggestion of an accumulation-like behavior for the initiation process. The potential for DNA replication (Fig. 3), the approximate positions of the maximal rate of cell division (Figs. 5, 6, 7), and the elution pattern of cells grown in BU-medium are all in agreement with the model. Indeed, periods of low, normal, and enhanced rate of cell division can be detected in at least two of the experimental elution curves in Fig. 5 *b* (after 30 and 45 min of growth in BU-medium). However, the shapes of the peaks in the elution curves and the time intervals between them did not always correspond precisely to the theoretical calculations. This was probably due to limitations of the experimental technique (see Helmstetter, 1967) and to the assumption that the treatments were selective for DNA replication. In addition, any delay in attaining the definite rate of DNA replication after a transfer of media, and any dispersion in the times for initiation and C and D would alter the positions of the peak and obscure any fine structure in them.

The accumulation-like behavior of initiation has been noted by a number of investigators (Nakada, 1960; Maaløe, 1963; Pritchard and Lark, 1964; Lark and Lark, 1964; Hayes, 1965; Pritchard, 1966; Maaløe and Kjeldgaard, 1966). In particular, Maaløe and Rasmussen (1963) presented evidence that the capacity for initiation is linked to the cellular mass and that a "built-in" control mechanism restores DNA/mass ratio in cultures starved temporarily of thymine. Pritchard and Lark (1964) suggested that thymine starvation initiated a new cycle of replication but only at the origin of one of the two partial replicas. Recently, Kallenbach and Ma (1968) reported enhanced initiation on only one of the arms of the replicating chromosome after 30 min of thymine starvation in *Bacillus subtilis*. These observations could also be consequences of symmetrical initiation at both origins in half of the cells in the population. Since in the experiments of Kallenbach and Ma (1968), the generation time was 40–50 min, less than 70% of the cells would be expected to initiate replication after 30 min of thymine starvation.

A synchronous division following restoration of thymine to thymine-starved

cultures was first reported by Barner and Cohen (1956). Donachie and Hobbs (1967) observed a recovery of viability in thymine-starved cultures restored to thymine prior to plating. My results are consistent with these findings since a synchronous division was observed when thymidine was added to starved cultures.

The results are consistent with enhanced initiation of chromosome replication after substitution of 5-bromouracil in place of thymine in exponentially growing cultures (Abe and Tomizawa, 1967; Wolf et al., 1968; Yoshikawa and Haas, 1969). However, my results cannot be explained by a sudden "induction" of multiple replication forks in the chromosome of all the cells after a given period of selective inhibition of DNA synthesis. A complete initiator unit would be attained at different times in different cells of an exponentially growing culture. The increase in potential for DNA synthesis after treatment with 5-bromouracil strongly suggests that the initiation of chromosome replication in a culture reflects the integral of the initiation processes in cells of different ages.

In summary, the results support the existence of a regulatory mechanism in bacterial cells with the following properties: a fixed amount of an initiator substance must be synthesized for the induction of a round of chromosome replication and division follows $C + D$ min later. The accumulation-like behavior of the initiation process offers a common explanation of the reports of an increased rate of DNA synthesis following restoration of DNA replication after a period of selective inhibition by thymine starvation (Nakada, 1960; Maaløe and Rasmussen, 1963; Pritchard and Lark, 1964; Kallenbach and Ma, 1968), ultraviolet light (Swenson and Setlow, 1966), or by other agents (Boyle et al., 1967). It accounts for and interprets the synchronous division observed by Barner and Cohen (1956).

APPENDIX A

Potential for DNA Replication in Cells with Known Chromosome and Initiator Contents

The potential for DNA replication in a cell can be determined by measuring the actual amount of DNA synthesis by that cell during a specific period of incubation in growth medium. First, I will consider the amount of DNA which would be synthesized by a newborn glucose-grown cell (C = 40 min) during a 40 min incubation period in glucose minimal medium. This will be followed by similar calculations for newborn cells incubated in BU-medium.

A newborn cell in glucose minimal medium contains one half-replicated chromosome and 0.5 units of initiator per origin (see text). During the first 20 min of growth in medium in which C = 40 min, the single replication point proceeds to the terminus of the chromosome resulting in the synthesis of 0.5 genome equivalents of DNA. During this time, 0.5 units of initiator have accumulated per origin. The cell now contains one unit of initiator per origin and inaugurates a new round of replication on both chromosomes. During the next 20 min, one genome equivalent of DNA synthesized, and therefore, at the end of the 40 min period, the total amount of DNA synthesized per cell is 1.5 genome equivalents.

The potential for DNA synthesis after incubation in BU-medium was found experimentally by measuring the amount of DNA formed by cells eluted from membrane filters (newborn cells) during growth in BU-medium (see text). Since the eluted cells were all newborn, the

chromosome configuration and the initiator content of samples taken from the membrane at any time can be calculated (see, for example, Table I). As examples, I will describe the calculations for samples taken at two different times from a membrane:

(*a*) The cells which divided (eluted) after D (20) min of elution with C = 120 min must have just completed a round of chromosome replication at the start of elution and begun a new round of replication. During elution, the new replication points progress along 2/12 of the chromosome (C = 120 min) and 0.5 units of initiator are synthesized per origin. If these cells were now incubated in medium in which C = 40 min, the total amount of DNA synthesized can be calculated as follows. The round of replication with be completed in $10/12 \times 40$ min. During this process, 10/12 genome equivalents of DNA are synthesized. During the first 20 min, 0.5 units of initiator will have accumulated per origin and the cell would inaugurate replication on both origins at this time. During the following 20 min, one genome equivalent of DNA is synthesized. The total amount of DNA synthesized becomes $1 + 10/12$ units of DNA per cell.

(*b*) Cells which divide (elute) after 50 min of elution in C = 120 min must have completed a round of replication at 30 min after the start of elution. Thus, these cells must have contained a single, 9/12-replicated chromosome and 0.75 unit of initiator per origin at the beginning of elution. They would initiate a new round of replication at 10 min of elution. Therefore, at division the newborn cells contain a single chromosome which is 4/12-replicated and 1.0 units of initiator per origin.

During subsequent incubation in medium in which C = 40 min a new round of chromosome replication will be inaugurated immediately on both origins so that the chromosome contains three replication points and 0 units of initiator per origin. The original replication point proceeds to the terminus in $8/12 \times 40$ min, and the two new replication points traverse the entire chromosome in 40 min. Thus, total amount of DNA per cell synthesized during a 40 min period being equal to $2 + 8/12$ genome equivalents.

APPENDIX B

Elution Pattern of a Culture Incubated for 45 Min in Medium in Which C = 120 Min

As an example of the derivation of the theoretical elution curves given in Figs. 5 *a* and 6 *a*, the construction of the 45 min curve in Fig. 5 *a* will be described in detail (Fig. 8). In this experiment, an exponentially growing population (C = 40 min) was shifted to C = 120 min for 45 min, bound to a membrane, and eluted with medium in which C = 40 min. If the culture had not been bound to the membrane, but the medium shifts had taken place, the concentration of cells in the culture would have increased as shown in the upper curve in Fig. 8. Time 0 in the figure is the time when the cells were returned to C = 40 min. The curve was obtained through calculations similar to those indicated in Table I and described in detail in the text (Fig. 4).

The lower curve shows the concentration of cells in the effluent if the culture had been bound to a membrane at 0 min. This was obtained by differentiating the upper curve and considering the age distribution of the bound populations as follows: (1) The membrane elution pattern gives the rate of cell division of a culture continuously deprived of one of the two daughter cells formed by division. Each time the rate of cell division changes in the culture (top curve) the rate of release of newborn cells increases or decreases abruptly (bottom curve). (2) An exponential cell population has twice as many cells of age 0.0 as cells of age 1.0. Therefore, a sudden decrease in the elution pattern is observed after all of the cells on the membrane have divided once (first arrow) and twice (second arrow) since the number of dividing cells decreased by a factor of two at these times.

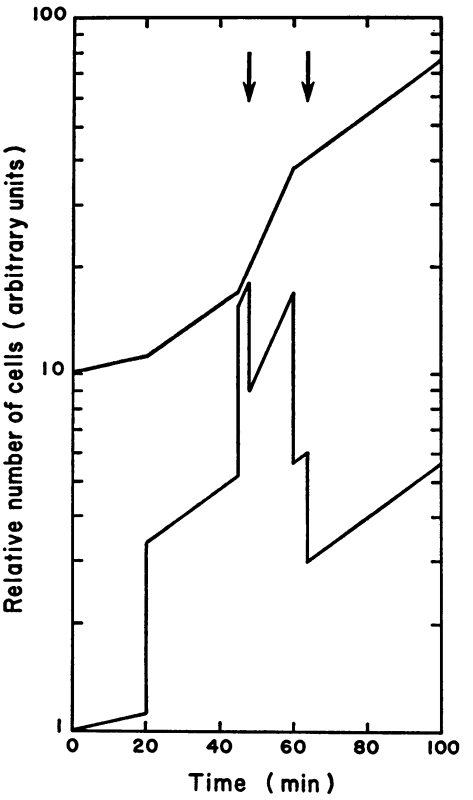


FIGURE 8 Theoretical number of cells in a culture restored to $C = 40$ min after 45 min incubation at $C = 120$ min. The top curve shows the relative cell concentration, the bottom one the calculated elution pattern.

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