# Synchronization of Bacteria by a Stationary-Phase Method[1]

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## ABSTRACT

CUTLER, RICHARD G. (University of Houston, Houston, Tex.), AND JOHN E. EVANS. Synchronization of bacteria by a stationary-phase method. J. Bacteriol. 91:469–476. 1966.—Cultures of *Escherichia coli* and *Proteus vulgaris* have been synchronized, with a high percentage phasing, in large volumes and at high cell densities by a method which takes advantage of a tendency of cells to synchronize themselves when entering the stationary phase of growth. The method consists of growing the bacteria to an early stationary phase, harvesting them quickly under minimal conditions of stress, and inoculating them into fresh medium at a dilution of about sevenfold. Cellular division is then partially synchronized. Four-generation cycles of a high percentage of phasing are obtained by repeating this procedure on the partially synchronized culture. Deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein analyses were made throughout all phases of the growth curve. Advantage has been taken of this method of synchrony to isolate selected segments of the bacterial genome in significant amounts. A working hypothesis to explain the synchrony suggests that the unfavorable conditions of growth as the bacteria near the stationary phase are detected by a decrease in the amino acid pool size, and that this results in a gradual decrease of DNA transcription activity through the inhibition of RNA polymerase by transfer RNA. The synchronizing method may be unique in producing cultures that grow both in cellular division and in genomic synchrony.

A culture of cells dividing synchronously allows an investigator to study the biochemical or structural nature of a single cell by providing enough cells at the same stage of cellular growth to make analysis possible. The synchronous bacterial system becomes of even greater value when the replication state of the genome can be correlated with the stage of cellular division. [In this paper, a cellular division and genomic synchrony culture is defined as a culture in which the deoxyribonucleic acid (DNA) replication cycle is initiated at the same time, from the same single site, and in the same direction for all the cells during each cellular division cycle.] This type of synchrony was shown for *Escherichia coli* K-12 Hfr (23, 24) and *Bacillus subtilis* W 23 (22, 25, 32–35). With synchronized cultures, the study of transformation, conjugation, DNA transcription activity, periodic protein synthesis, and enzymatic activity, to mention a few applications, can now be made not only in relation to the cellular di-

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vision cycle but also in relation to the physical state of the genome replication cycle.

The progress of research, however, has been limited in the absence of adequate means of synchronizing cultures (6, 10, 21). This paper presents a successful method that is simple and probably widely applicable. Cultures with a high percentage of phasing (36) at densities of about $5 \times 10^8$ cells per milliliter have been produced with no special equipment or auxotrophic bacteria required, and the volume is limited only by the size of the investigator's standard cultivation and harvesting facilities. The method is easy to reproduce, and bacteria appear to be free from unbalanced growth conditions in the mid-log phase of growth.

The bacteria are synchronized by allowing them to grow to the stationary phase from which, on inoculation into fresh medium, division synchrony occurs. Although similar physiological methods of synchronization have previously been used for protozoa (2), yeast (29), and bacteria (14, 31), they have not been used widely because of the small percentage of phasing indicated and possible

disturbances of balanced growth conditions. More recently, bacteria taken from the stationary phase have been utilized to synchronize the DNA replication cycle (32–35) and the cell division cycle (22) in cultures of *B. subtilis* W 23.

The synchronization method given in this paper was suggested by the results of the physiological and morphological studies of bacteria by Henrici (11), Hershey and Bronfenbrenner (13), Hershey (12), Winslow and Walker (30), and Huntington and Winslow (15). Their work indicated that bacteria (especially when grown in a minimal medium) are in a more homogeneous physiological and morphological state in the early stationary phase than in the mid-log phase of the growth cycle. This synchronization concept led first to the synchronization of *Proteus vulgaris* (paper presented by the authors at the Fifth National Congress of Microbiology, Mexico City, 1964) and then to the synchronization of the K-12 Hfr, B/r, and K-12 F⁻ strains of *E. coli*.

This paper presents the experimental conditions used to synchronize *P. vulgaris* and *E. coli* and two applications of the method. These are the measurement of the grams per milliliter and grams per cell of DNA, RNA, and protein throughout all phases of growth and the isolation of selected genome segments of *E. coli*. A working hypothesis is presented to explain how the synchrony is accomplished.

## MATERIALS AND METHODS

*Organisms.* *P. vulgaris*, phase B (1), was isolated from a urine sample, *E. coli* K-12 Hfr CS 101 *met⁻* was obtained from A. Pardee, and *E. coli* K-12 Hfr Ca 3000 *met⁻thy⁻thi⁻* was obtained from J. Cairns.

*Media.* Mineral salts [C medium of Roberts et al. (27)] at $pH$ 7.3 with 0.5% glucose were used for all experiments unless otherwise indicated. DL-Methionine (50 $\mu$g/ml), thymidine (10 $\mu$g/ml), thiamine (2 $\mu$g/ml), and bromodeoxyuridine (BUDR; 20 $\mu$g/ml) were used when indicated. For *E. coli* K-12 Hfr Ca 3000 *met⁻thy⁻thi⁻*, charcoal-purified Casamino Acids were added to 0.1% for the amino acid requirement. With this medium, the approximate generation time of an asynchronous culture at the mid-log phase is 45 min for *E. coli* and 70 min for *P. vulgaris*.

*Synchronization technique.* An isolated colony was grown overnight with 0.05% glucose, and 5 ml of the culture was used to inoculate 100 ml of medium in a side-arm flask. Optical density was measured in this flask with a Klett-Summerson photoelectric colorimeter with a no. 42 filter. The cells were harvested in the early stationary phase by centrifugation at 3,000 × *g* for 9 min in a 125-ml centrifuge tube under constant conditions at 37 C, and were inoculated into 750 ml of medium (about a sevenfold dilution) at 37 C under forced aeration of 1,800 cm³/min. The time interval from stationary phase to fresh medium was not more than 15 min. Optical

density of this culture was measured on 3-ml samples with a Beckman DU spectrophotometer at 420 m$\mu$. The cells were again harvested at the early stationary phase by the same procedure as before and were inoculated into 5,500 ml of fresh medium at 37 C (about a sevenfold dilution) under forced aeration of 3,600 cm³/min. Optical density of the culture was again measured on 3-ml samples with a Beckman DU spectrophotometer at 420 m$\mu$. The cells in this culture were about 70 to 90% phased throughout three- or four-generation cycles. The volumes used may be scaled up or down according to needs; however, the last flask may be inoculated at a lower cell density to obtain balanced growth conditions. The side-arm flask was incubated in a water bath at 37 C shaking at 240 rev/min, and the other flasks were in a stationary water bath at 37.5 C. In general, the optimal harvest points were one-half of the bacterial generation time after the culture enters the stationary phase as defined by optical density.

However, because of the variation of optimal harvest periods between species and strains, it was found to be best to optimize the conditions for each. The optimal harvest periods for *E. coli* and *P. vulgaris* strains are shown in the results.

It is not clear which factor(s) is growth-limiting when the stationary phase is initiated. Therefore, the growth conditions described here should be carefully followed, although more satisfactory conditions are likely to be found at a later time.

*Cell density determination.* Cell density was determined by the Petroff-Hausser chamber counting technique. Samples (1 ml) were collected every 5 min in tubes containing 0.05 ml of 40% formaldehyde and were stored at 4 C. From 300 to 600 cells were counted for each point. Cell density and morphology were followed in synchronous cultures with a lag of no more than 5 min. An approximation of per cent viability, made by calculating the cell density ratio between division steps, correlated well with actual viable counts.

*Biochemical analyses.* Samples (10 ml) were collected every 5 min for the analysis of DNA, RNA, and protein. The samples were immediately brought to 0.5 N with perchloric acid and were cooled to 4 C. DNA and RNA were extracted, and protein was precipitated by the method described by Burton (3). DNA was analyzed by Burton's modification of the Dische method (3), RNA by the orcinol method of Dische and Schwartz (7), and protein by the method of Lowry et al. (20).

*BUDR pulse labeling.* Samples were collected every 5 min. To keep the harvested cellular weight constant, the volumes of the samples ranged from 250 ml down to 80 ml. Each sample had thymidine replaced by BUDR within 2 min by filtration (9) under constant conditions at 37 C. After 20 min of incubation at 37 C, which labeled about one-tenth of the genome, the BUDR incorporation was stopped by the addition of one-third volume of C medium (without glucose) at 4 C containing 0.03 M KCN and 50 $\mu$g/ml of thymine.

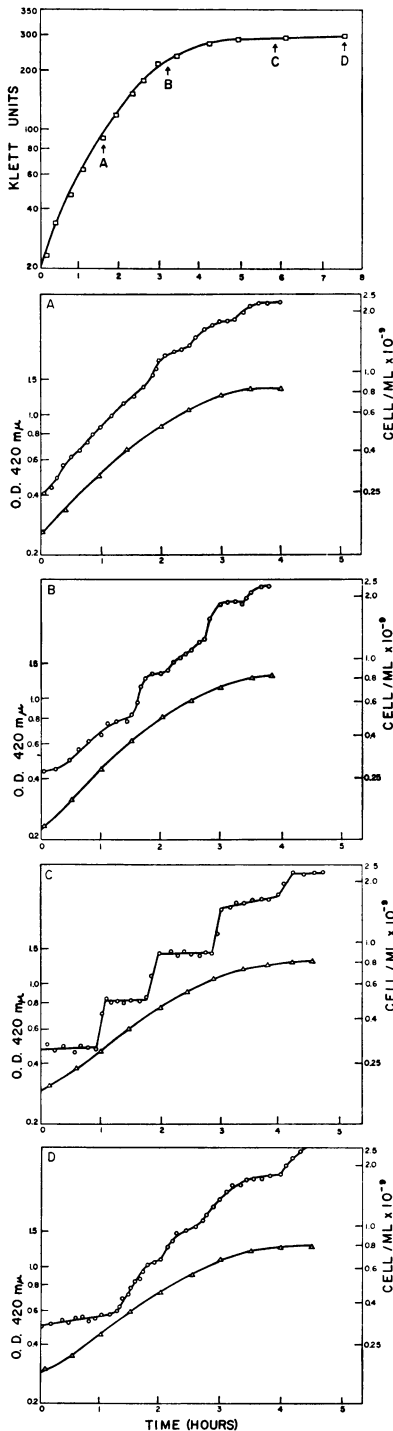The per cent recovery of cell mass after harvesting by filtration was approximately 90% and no change

in per cent viability was found. By assuming, then, that almost 100% of the cells will incorporate the BUDR when pulse-labeled, the percentage of heavy labeled DNA can be calculated by measuring the relative amount of hybrid and native DNA.

*DNA isolation.* Pulse fractions were centrifuged at $5,000 \times g$ for 15 min, washed, and suspended in 5 ml of a solution of 0.15 M NaCl and 0.1 M ethylenediaminetetraacetate (EDTA) (pH 7.3). Cells were lysed at 37 C for 5 min with 100 $\mu$g/ml of lysozyme and then were deproteinized by the addition of 50 $\mu$g/ml of pronase and incubation for 60 min at 37 C. Enzymatic activity was then stopped by adding sodium lauryl sulfate to 1% and heating for 10 min at 60 C. The mixture was cooled quickly to room temperature. This crude lysate was brought to a density of 1.72 g/cm³ by the addition of Harshaw solid CsCl and was centrifuged in an SW 39 model L rotor at 33,000 rev/min for 72 hr at 25 C. Two drops per fraction was collected from the needle-pierced centrifuge tubes at room temperature in tubes containing 0.5 ml of a solution of 0.015 M NaCl and 0.0015 M sodium citrate. Optical density of fractions was measured in a microcuvette at 260 m$\mu$ with a Beckman DU spectrophotometer.

## RESULTS

The results of four experiments to determine the optimal harvest time for the synchronization of *E. coli* K-12 Hfr CS 101 *met⁻* are shown in Fig. 1. The first indication that the cells were being synchronized was the appearance of slight synchrony steps as the cells entered the stationary phase. The cells became more synchronized as the harvest point neared the stationary phase, and then the synchronized condition began to decay as the harvest point entered the late stationary phase.

Figure 2 demonstrates the stationary-phase synchrony method with *P. vulgaris*. DNA, RNA, and protein were determined on samples from the synchronous culture of *P. vulgaris* in Fig. 2C. It was found that DNA, RNA, and protein increased steadily throughout the entire generation cycle, with only slight deviations at the beginning of the growth curve and at division intervals (Fig. 3). The maximal RNA and protein per cell in the mid-log phase correlated well with the phase of maximal physical size found for the bacteria. RNA, protein, and DNA synthesis were initiated in that sequence after the cells were inoculated into the fresh medium, and synthesis

FIG. 1. *Effect of harvesting Escherichia coli K-12 Hfr CS 101 met⁻ at different points of the growth curve. Four separate experiments are shown. Cells were first grown in 100 ml of medium in a side-arm flask and harvested at the different stages of their growth curves (points A to D on top diagram). Cells were then inoculated into a volume of fresh medium (1A to 1D) that would give each of the four experiments the same initial optical density. Symbols: □, Klett units; △, optical density at 420 m$\mu$; ○, cells per milliliter.*
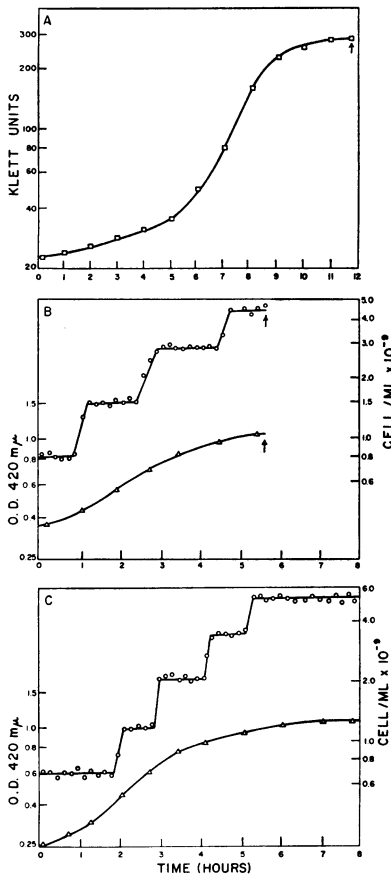
FIG. 2. *Synchronization of Proteus vulgaris. Cells were first grown in 100 ml of medium in a side-arm flask (2A), harvested at arrow, and inoculated into 750 ml of fresh medium (2B). The cells were then harvested at arrow and inoculated into 5,500 ml of fresh medium (2C). From this last flask, samples were taken for DNA, RNA, and protein analyses. Symbols: □, Klett units; △, optical density at 420 mμ; ○, cells per milliliter.*



FIG. 3. *Results of the DNA, RNA, and protein analyses on Proteus vulgaris synchronous culture. The grams per cell of DNA, RNA, and protein is calculated from the average values of cells per milliliter and DNA, RNA, or protein per milliliter. Symbols: —, cells per milliliter; ○, grams per milliliter; △, grams per cell.*

stopped immediately after the last division, which occurred in the stationary phase. Although a balanced growth condition is not indicated in this experiment, it can be obtained in the mid-log phase if the initial cell density is one-tenth as great.

In the experiment to isolate selected segments of *E. coli* genome, two main conditions were required of a synchronized culture. First, the genome was required to be fractionated into about 10 sections, with at least 20 μg of each needed for physical studies. To furnish this amount of DNA, a large cellular yield from the synchronized culture was necessary. Second, the cells had to be in cellular division and genomic
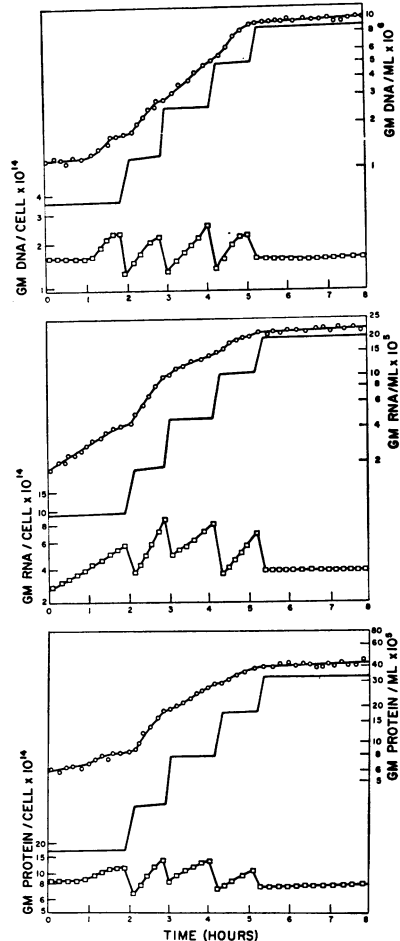
synchrony. This allowed all the cells to be heavy-labeled in the same area of the genome for a given time interval at selected periods of their DNA replication cycle. The heavy-labeled sections of DNA could then be isolated from the remaining native DNA by CsCl density gradient centrifugation.

The stationary-phase synchrony method easily satisfied the first requirement. For the second requirement, Cairns (4) showed that the Hfr strain used in this experiment has sequential DNA replication from a single growth site, and Nagata (23, 24) showed cellular division and genomic synchrony with a similar strain. Pre-

liminary results in our laboratory indicated that the stationary-phase synchrony method synchronizes this strain to grow in cellular division and genomic synchrony.

Figure 4 shows the growth curves for this Hfr strain and the points at which 25 different samples of cells were collected and pulse-labeled with BUDR. A 10-min amino acid and glucose starvation period at the time of inoculation of the 5,500 ml of medium ensured a better cellular division and genomic synchronization. This brief starvation period possibly allowed those cells which had not completed their DNA replication cycle to
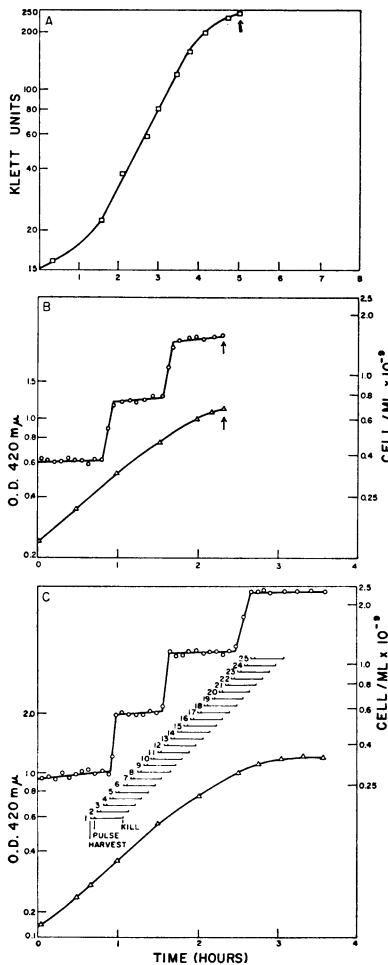


FIG. 5. *Optical density profile of collected fractions from the CsCl density gradient of one of the BUDR pulses. From each gradient tube, approximately 25 μg of the genome that was replicated during the interval of 5/11 to 6/11 of the replication cycle was isolated (hybrid band).*

complete it, while holding back the cellular growth and reinitiation of the DNA replication cycle in the cells which had already completed this cycle (18, 19).

Figure 5 shows the isolation of the hybrid DNA that was replicated during the 5/11 to 6/11 period of the genome replication cycle. Segments of the genome prepared in this manner were used to determine their relative transcription activity in relation to the cell generation cycle (paper presented by the authors at the Ninth Annual Biophysical Society Meeting, San Francisco, 1965).

## DISCUSSION

These experiments indicate that a bacterial culture becomes synchronized physiologically and morphologically when nearing the stationary phase, and if advantage is taken of this synchronization phenomenon a useful synchronously growing culture can be obtained. *P. vulgaris* and *E. coli* K-12 Hfr, K-12 F−, and B/r have been synchronized successfully by this method. No other strains have been tried

The appearance of partial synchronization about two generation cycles before the onset of stationary phase in Fig. 1A suggests that these synchronization forces may not only be able to synchronize a random culture but that they could also stabilize a previously synchronized culture. This may account for the high degree of synchrony obtained for three or four generations, in spite of the wide fluctuation of individual cell generation times that Powell (26) found in random cultures.

It now seems difficult to obtain a culture in which the average cell is 50% through its generation cycle. The average cell in an asynchronous, logarithmically dividing culture is 47% through



FIG. 4. *Synchronization of Escherichia coli K-12 Hfr Ca 3000 mer⁻thy⁻thi⁻. The synchronization procedure was similar to that used for Proteus vulgaris. The resultant synchronous culture (4C) had 25 samples collected every 5 min (Harvest), pulse-labeled with BUDR (Pulse), and then the BUDR incorporation was stopped (Kill). Symbols: □, Klett units; △, optical density at 420 mμ; ○, cells per milliliter.*
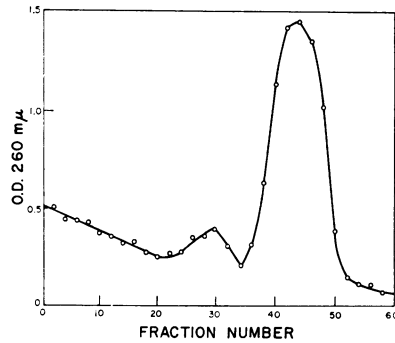
its generation cycle (5). This percentage is apparently a maximal limit, and decreases to zero as the culture enters the stationary phase. Thus, in any study with bacterial cultures, there is the possibility of unintentionally obtaining a culture with a high percentage of phasing.

The working hypothesis for the tendency of cells to synchronize themselves when nearing the stationary phase of growth has been based on the relationship between amino acid synthesis and DNA transcription activity. Figure 6 illustrates some of the features of this hypothesis. These are the replicon (16), which controls the initiation of DNA replication by way of the initiator protein, and the transfer RNA (tRNA), which combines with the amino acids or inhibits the RNA polymerase (8, 17, 28) upon which all DNA transcription is dependent. When a random culture nears the stationary phase, conditions are becoming less favorable for the cell to maintain its metabolic rate. Thus, the culture is entering step-down growth conditions, but in infinitely small steps. The size of the amino acid pool will then decrease as the total metabolism of the bacteria is lowered. This leaves excess unactivated tRNA to complex with RNA polymerase, which results in the lowering of DNA transcription activity. The rate of protein synthesis is then decreased slowly with the decrease in messenger RNA concentration. This further decreases the rate of amino acid synthesis as the feedback loop is completed  As a result, cells near the stationary phase are growing faster than those already in it, and a bunching effect is produced which soon synchronizes the entire culture. A homogeneous population of cells are gathered in the early sta-

tionary phase, which have DNA transcription and replication completely repressed before the nutrients in the medium become entirely exhausted. Here the cells have the highest ratio of DNA to protein and DNA to RNA, and because the time of the generation cycle is relatively constant throughout the growth curve the cells have decreased to about one-fourth the size of the log-phase cells.

When cells are taken from this phased condition and inoculated into fresh medium, the inhibition of RNA polymerase would then be released as newly synthesized amino acids become activated with the existing tRNA. The cells then simultaneously start the transcription of DNA, followed by the synthesis of protein, and then by the replication of DNA. However, if the stationary-phase cells were allowed to grow older before harvesting, the concentration of messenger RNA and the necessary enzymes to synthesize amino acids would further decrease in proportion to their different half-lives. This would result in a longer lag time needed to release the inhibition of RNA polymerase and a less uniform initiation of transcription activity in the fresh medium. In the deep stationary phase, cells would begin to lyse, allowing others to divide, and resulting in the further decay of the homogeneous state.

*E. coli* strains K-12 Hfr H *met⁻* and K-12 CS 101 *met⁻*, which are relaxed in their DNA transcription control (28), were found to synchronize with the same percentage of phasing as the stringent B/r *E. coli* strain. Thus, stringent control over DNA transcription activity is not required for the cells to become synchronized.

*E. coli* K-12 Hfr and *B. subtilis* W 23 are the only bacteria that have been shown to grow in cellular division and genomic synchrony, and yet both of the synchrony techniques used for these bacteria involved growing the cells to a stationary phase before inoculating them into fresh medium. For example, when the selection method of synchronization used by Nagata (24) and Masters et al. (22) was repeated without the filtration step, the cells were found to be partially synchronized. This synchronization, which resulted from the cells being harvested from the stationary phase, could account for some of the success of the selection method.

There is also the possibility that the *E. coli* K-12 Hfr strains and *B. subtilis* W 23 strain were found to grow in cellular and genomic synchrony only because they were harvested from the stationary phase. The increasing concentration of tRNA would stop the replicator from being initiated at some critical point in the early stationary phase for the Hfr and W 23 bacterial
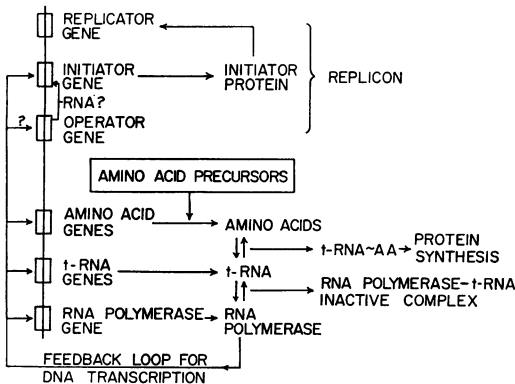


FIG. 6. *Schema used to explain a hypothesis of how the stationary phase synchrony method may work, showing the interrelationship of protein synthesis, DNA transcription activity, and replicon function with the concentration of amino acid precursors and the inhibition of RNA polymerase by tRNA.*

strains. Thereafter, the DNA replication cycle would be able to be completed but not to be reinitiated. The F⁻, B/r, and W 168 strains, which are thought to grow randomly with respect to the cell division and genomic replication cycle, would still be initiating the replicator under these conditions, although severe amino acid starvation may stop the reinitiation (18, 19). Replication would be terminated at any stage of the cycle when necessary precursors become depleted.

However, the other phased physiological and morphological characteristics at the early stationary phase would enable both bacterial types to grow in division synchrony when inoculated into fresh medium; but the Hfr and W 23 strains will be in cellular division and genomic synchrony, whereas the F⁻, B/r, and W 168 strains will have a random DNA replication cycle with cellular division synchrony. This random DNA replication cycle with respect to cellular division synchrony does not require more than one replicon initiator site per genome if the bacteria are multinuclear in multiples of two. The cells could then divide after doubling their total DNA content at random, uncompleted states of the DNA replication cycle. The realization of the synchronizing mechanisms operating in the stationary-phase synchrony method and the differences between these two types of bacteria may further our understanding of how the DNA replication and cell division cycle is regulated.

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