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Chromosome Replication in *Salmonella typhimurium*[1]

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The replication of the *Salmonella typhimurium* chromosome was studied. As with *E. coli* 15T⁻, replication was sequential. After amino acid starvation, replication proceeded from a unique and heritable region of the chromosome. 5-Bromouracil, when substituted for thymine, did not disturb the sequence of replication nor did it initiate extra replication cycles. By labeling the origin and the terminus of the chromosome with ^{3}H- and ^{14}C-thymine, respectively, it was possible to determine that the rate of chain elongation decreases as the growth rate decreases. No gap in the replication cycle could be observed.

The sequential replication of the bacterial chromosome has been demonstrated in *Escherichia coli* by physical and genetic techniques (1, 4, 8, 11, 13, 14, 18). These studies led to the conclusion that replication is initiated at a unique position on the chromosome. Replication ceases at this locus after starvation for amino acids, which allows a replication cycle to be completed but prevents reinitiation of a new cycle (9; R. Bird and K. G. Lark, *in press*; B. Wolf et al., Cold Spring Harbor Symp. Quant. Biol., *in press*). Similar results have been obtained in studies of *Bacillus subtilis* (2, 17).

We extended our previous physical studies of the *E. coli* chromosome to *Salmonella typhimurium*. This organism has been demonstrated to form genetic hybrids (19) with the deoxyribonucleic acid (DNA) of *E. coli* and its physiology has been examined in detail (12).

The present study demonstrates that most aspects of chromosome replication in *Salmonella* and *Escherichia* are similar.

MATERIALS AND METHODS

Bacterial strains. Five strains of *S. typhimurium* were obtained from A. Eisenstark. Strain 1660H is a methionine, arginine, tryptophan, and thymine quadruple auxotrophic mutant. The other Hfr strains (3612, 3613, 3632, and 3660) all require histidine and thymine. Their mating types and growth rates in different media are given in Table 1. *E. coli* 15T⁻ (557) has been described previously (11, 14).

Media and supplements. The organisms were grown with aeration at 37 C in either minimal or Casamino Acids (Difco)-supplemented M9 synthetic medium ($NaHPO_4$, 0.7%; $MgSO_4$, 0.02%; KH_2PO_4, 0.3%; NaCl, 0.05%; NH_4Cl, 0.1%; glucose, 0.4%; $CaCl_2$, 0.002%; *p*H 7 to 7.2). Strain 1660H was supplemented with 20 μg of thymine per ml, 30 μg of L-methionine per ml, 14 μg of L-tryptophan per ml, and 34 μg of L-arginine per ml. Strains 3612, 3613, 3632, and 3660 were supplemented with 20 μg of thymine per ml and 20 μg of L-histidine per ml. When used as a supplement, the final concentration of Casamino Acids was 0.1%.

^{13}C-glucose and $^{15}NH_4Cl$ medium was M9 medium in which the glucose and NH_4Cl were replaced by 0.1% ^{13}C-glucose and 0.05% $^{15}NH_4Cl$. Cells grown in this medium were not supplemented with Casamino Acids.

The concentrations of thymine and 5-bromouracil (5-BU) were 10-fold greater than those required for exponential growth to a cell density of 10^9 cells per ml. When ^{3}H-thymine, ^{14}C-thymine, or ^{14}C-5-BU were used, the concentration of thymine or 5-BU was usually reduced to 2 μg per ml (nonradioactive 5-BU was used at a concentration of 20 μg per ml when it replaced thymine).

Nutrient agar (7) was used for bacterial colony counts.

Changes of media were accomplished by collecting the cells on a Schleicher and Schuell membrane filter (coarse A), washing the filter with prewarmed medium at 37 C, and resuspending in the new medium. We recovered 75 to 95% of the cells by this procedure which took 1 to 2 min.

Measurement of cell numbers. Cells were counted in a Coulter counter (model B) with a 30-μm orifice, a current setting of 3, and a maximum gain and lower threshold of 10.

On occasion, cells were also counted microscopically in a Petroff-Hauser counting chamber.

Viable cell counts were determined by colony counts (7).

Measurement of DNA synthesis and determination of cellular DNA content. Incorporation of radioactive thymine or 5-BU into DNA was measured by collecting 0.1-ml samples on membrane filters and washing these either with hot (90 C) water (6) or with cold (4 C) 5% trichloroacetic acid (both methods yielded

[1] This research has been submitted by Hilary Chan as part of a thesis in fulfillment of the requirements for the Ph.D. degree.

TABLE 1. *Growth rates and mating types of S. typhimurium strains*[a]

Strain	Generation time (min) in		Mating type
	M9	M9 + Casamino Acids	
1660H.....	65	42	F^-
3612.....	60	37	HfrA
3613.....	67	42	HfrA
3632.....	67	64	HfrB3
3660.....	67	66	HfrA

[a] Growth rates were determined as an increase in cell number in the Coulter counter. Mating types are those described by Sanderson (15).

identical results). The dried filters were counted in a scintillation counter.

DNA synthesis was measured, after the incorporation of nonradioactive 5-BU or of the heavy isotopes ^{13}C and ^{15}N, as the per cent of hybrid-density DNA synthesized.

The cellular DNA content was also determined by the diphenylamine reaction (3).

Analysis of DNA samples by CsCl density gradient centrifugation. Samples (10- or 20-ml) of bacteria were pipetted quickly onto 6 g of crushed frozen M9 medium (−50 C) and were centrifuged; the resulting pellet was frozen. The frozen pellet was subsequently thawed and treated for 10 min at 37 C with 0.75 ml of a solution containing 10 μg of lysozyme in 0.02 M ethylenediaminetetraacetic acid (EDTA). A 1-ml amount of a 1% solution of sodium lauryl sulfate in 0.02 M EDTA was then added, and incubation at 37 C was continued for another 15 min. A 2.25-ml amount of a 1.2% solution of recrystallized papain (18) was then added, and, after mixing, the sample was further incubated at 37 C for 20 min.

For analysis of the total light- or hybrid-density DNA, 0.32 ml of this crude lysate was diluted to 1 ml with water and CsCl to give a density of 1.75. The mixture was centrifuged for 16 to 20 hr at 42,040 rev/min in a model E Spinco analytical ultracentrifuge. Pictures were taken and analyzed with a Joyce and Loeble densitometer. The light- or hybrid-density types of DNA were calculated from the areas under the densitometer tracings of the corresponding peaks.

To obtain the distribution of radioactive material present as light- or hybrid-density types of DNA, 3.2 ml of the crude lysate was mixed with 4.36 g of CsCl. The mixture was centrifuged at 40,000 rev/min for approximately 45 hr in a 50 Ti angle rotor in a preparative Spinco ultracentrifuge. The centrifuge tubes (Beckman polyallomer) were punctured and fractions were collected in tubes containing 1 ml of water. The fractions were then chilled, 1 ml of a 10% trichloroacetic acid solution was added, and the contents were filtered through glass filters (Reeve-Angel). The radioactivity of the dried filters was measured. In experiments with 5-BU, 25 ten-drop fractions were collected, whereas when ^{13}C-glucose-$^{15}NH_4Cl$ was used as a density label, 55 four-drop fractions were collected. Figure 1 presents the radioactive profiles obtained after centrifuging DNA density labeled with 5-BU or $^{13}C^{15}N$.

Counting of radioisotopes. Radioisotopes of ^3H-methyl-thymine (10 μc per mmole), thymine-2-^{14}C (30 mc per mmole), and 5-bromouracil-2-^{14}C (30 mc per mmole) were obtained from New England Nuclear Corp. The radioactive samples were counted in either a Packard Tri-carb liquid scintillation spectrometer or a Beckman model 200 scintillation counter.

Samples on membrane or glass filters were counted in an organic scintillation fluid consisting of 3 g of 2,5-diphenyloxazole and 300 mg of 1,4-bis-2 (4-methyl-5 phenyloxazolebenzene) dissolved in 1 liter of toluene.

Autoradiography. Samples of cells to be autoradiographed were washed on membrane filters until free from medium radioactivity and were centrifuged; a drop of the concentrated suspension was placed on the surface of a partially dried nutrient agar plate. As soon as the drop had dried, impression smears were made on a glass slide and the slide was dipped, in succession, through two beakers of 5% trichloroacetic acid at 4 C and through three beakers of distilled water also at 4 C.

The slides were coated with Kodak NTB 2 film emulsion at 50 C and then dried. They were placed in Bakelite slide boxes and kept in a cool, dry, dark container while being exposed.

After developing the slides in a Kodak D19 developer for 5 min and fixing for another 5 min, they were dried and examined by phase contrast microscopy (× 500). Fields with well-isolated cells (between 24 and 40 per field) were scored. All of the cells in a single field were counted to avoid subjective selection of material.

RESULTS

Growth and labeling of S. typhimurium. Our ultimate purpose in these experiments was to describe chromosome replication in *S. typhimurium*. Before doing this, however, it was necessary to describe the physiology of this organism pertinent to such experiments.

With the exception of a few experiments, strain 1660H was used throughout this study.

This strain grew in supplemented M9 medium with a generation time of 65 min. When Casamino Acids were added, the generation time decreased to 42 min. We found DNA contents of 12.2×10^{-15} and 9.7×10^{-15} g per cell at the fast and slow growth rates, respectively. These values are in good agreement with previous results on other *Salmonella* strains (16).

This difference in DNA content was accompanied by a difference in the average number of replication forks per cell. This can be seen in Fig. 2, which presents the results of an experiment in which cultures were allowed to incorporate a pulse of ^3H-thymine and then were grown in a nonradioactive medium. Cells were removed

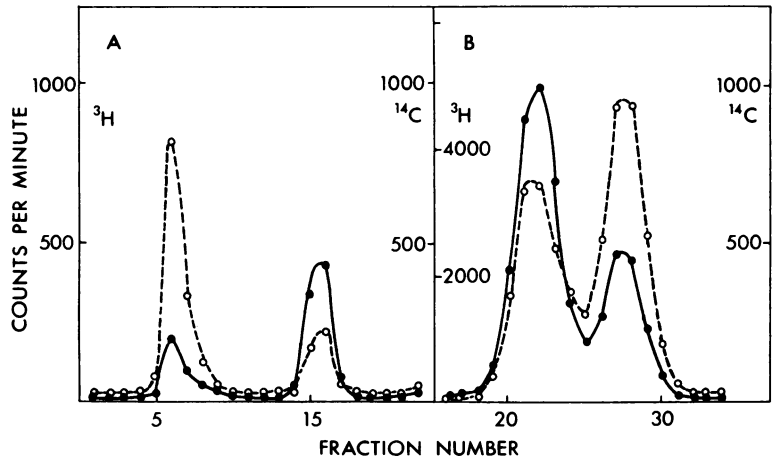

FIG. 1. *Density gradient profiles of DNA after centrifugation in a Ti 50 angle rotor. Samples were centrifuged at 40,000 rev/min. (A) DNA from cells grown in 5-BU, 40 hr of centrifugation; (B) DNA from cells grown in ^{13}C-glucose and $^{15}NH_4Cl$, 60 hr of centrifugation. The hybrid density peaks are on the left. Symbols: (○) ^3H; (●) ^{14}C.*

after 0, 1, 2, 3, or more generations of growth and autoradiographs were prepared. After a long exposure, most of the cells were either intensely labeled or unlabeled, indicating that the radioactivity was conserved in a few units which were distributed into daughter cells. Unlabeled cells appeared between the first and second generation in unsupplemented medium and between the second and third generation in Casamino Acids-supplemented medium. Extrapolation to the time of labeling [(0) generation] indicated the presence of two to three labeled units in the slowly growing cells and four to five units in the rapidly growing cells. These data indicated an average of 1.2 replication forks per cell in cultures with a 65-min generation time and 2.2 replication forks in cultures with a 42-min generation time.

In most of the experiments to be described, density labeling of DNA was required. To minimize expense and to increase the separation of hybrid- and light-density types of DNA, 5-bromouracil was used as a density label in place of thymine. Incorporation of this analogue was poor, but could be improved by the addition of deoxyguanosine to the medium (Fig. 3). Pyrimidine nucleosides, such as uridine or deoxycytosine, failed to improve 5-BU incorporation. In all subsequent experiments with 5-BU, deoxyguanosine was present. Although cells lose viability during growth in 5-BU, we did not observe a decrease in cell number (H. K. Chan, Ph.D. Thesis, Kansas State Univ., Manhattan, 1968). At present, we have no evidence to indicate that 5-BU induces a prophage, a phenomenon observed after thymine starvation of 1660H (J. M. Aldrich, M.S. Thesis, Kansas State Univ., Manhattan, 1967).

Figure 4 compares the replication of DNA when 5-BU is substituted for thymine in rapidly and slowly growing cultures. Replication was measured as the conversion of light- into hybrid-density DNA. After a short initial lag, replication proceeded in 5-BU, although the experiments in unsupplemented M9 indicated that replication in 5-BU was slower than in thymine. A lag also occurred before replication was observed in $^{13}C^{15}N$ media. These initial lags suggested the existence of an intracellular nucleotide pool which delays the appearance of hybrid-density DNA. Such a pool could influence the incorporation of a pulse of radioactive thymine. To test the extent of this effect, cultures of 1660H were exposed to ^3H-thymine for one-eighth of their generation period and then were transferred to nonradioactive medium. The further incorporation of ^3H-thymine was followed. Incorporation of radioactive material continued for 10 or more min in thymine medium and for 20 min in 5-BU medium (Fig. 5). This belated incorporation accounted for as much as 30% of the acid-insoluble radioactive material or 3 to 4% of the total DNA content of the bacteria.

It is interesting to note that less residual incorporation occurred in the absence of required amino acids.

In summary, 1660H incorporated 5-BU in the presence of deoxyguanosine. However, replication in the presence of the thymine analogue was slower than in thymine. Conversion to hybrid DNA was delayed for about 20 min upon trans-

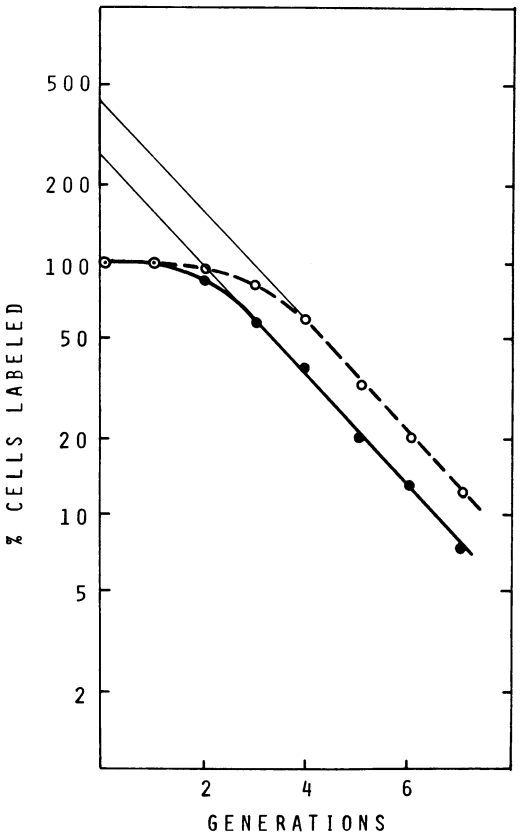

FIG. 2. *Segregation of radioactive DNA during growth of S. typhimurium. Exponential cultures of S. typhimurium 1660H in M9 (●) or Casamino Acids-supplemented M9 (○) were labeled with a pulse of ^{3}H-thymine (100 μc per 2 μg per ml) for one-eighth of their generation times. They were then washed and transferred to nonradioactive medium. The cultures were diluted during subsequent growth to maintain the cell titer between 10^{8} and 2×10^{8} cells/ml. Cell division was followed in the Coulter counter and samples were taken immediately or after the first, second ------ (broken line) or seventh doubling of the cell number. Autoradiographs were prepared, exposed for 16 days, developed, and examined for the per cent of radioactive cells (those with one or more grains). The majority of the labeled cells contained 10 or more grains. Three-hundred labeled cells were examined for each sample.*

fer to 5-BU medium when compared to 10 min after transfer to $^{13}C^{15}N$-thymine medium. This delay may be due to an intracellular pool of thymine and other nucleotides.

Sequential replication of the Salmonella chromosome. Exponential cultures of 1660H were grown in ^{14}C-thymine for several generations; then they were pulse-labeled with ^{3}H-thymine and transferred to 5-BU media. Replication of the pulse-labeled DNA (^{3}H) and of the total DNA (^{14}C) was measured at intervals as the conversion of radioactive DNA from a light to a hybrid density. If the chromosome replicated in a defined sequence, the entire chromosome should be replicated, that is, the ^{14}C-DNA should be converted to a hybrid density, during the replication which occurs between the incorporation of the ^{3}H-thymine pulse into the chromosome and the eventual conversion of this ^{3}H-labeled DNA to a hybrid density. Experiments were carried out with rapidly and slowly growing cultures (Fig. 6). The results are similar to those obtained previously with *E. coli*. More than 80% of the pulse label remained to be replicated after 50% of the total DNA had already been replicated. This result is consistent with a sequential mechanism of replication if we recall (from Fig. 5) that our "pulse" of ^{3}H-thymine is probably incorporated over a period equalling 30 to 40% of the generation period and that utilization of 5-BU may be delayed by the presence of an intracellular thymine pool. Both of these effects tend to blur the replication of the ^{3}H-pulse with respect to the rest of the chromosome.

Another experiment demonstrated the sequential nature of chromosome replication in *Salmo-*

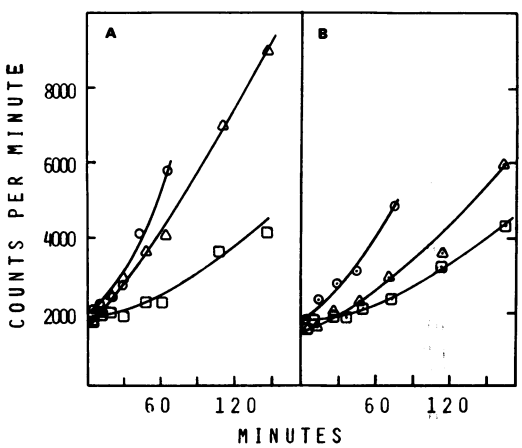

FIG. 3. *Incorporation of radioactive thymine or 5-BU under various growth conditions. Exponential cultures of 1660H were grown in Casamino acids—supplemented M9 (A) or in M9 (B) media. At a titer of 10^{8}, the cells were transferred to different media. Symbols: (○, ⊙) medium containing ^{14}C-thymine (5 μc per 2 μg per ml); (△, ◬) medium containing ^{14}C-5-BU (5 μc per 2 μg per ml), deoxyguanosine (20 μg/ml), and uracil (20 μg/ml); (□, ⊡) medium containing ^{14}C-5-BU (5 μc per 2 μg per ml). Samples (0.2 ml) were taken at intervals to measure the cold trichloroacetic acid-insoluble radioactivity.*

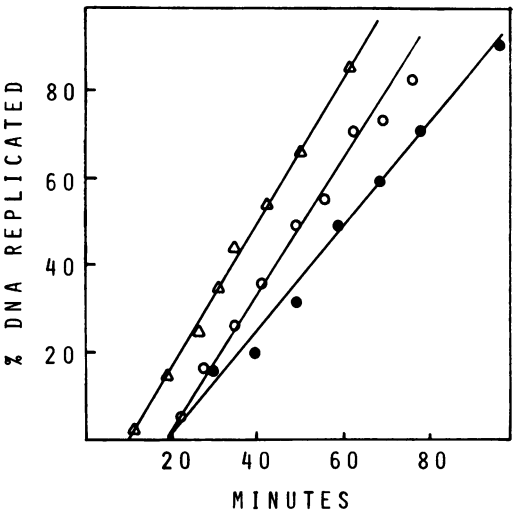

FIG. 4. *Replication of DNA in M9, M9 containing 5-BU, or Casamino Acids-supplemented M9 containing 5-BU. Exponential cultures in M9 were transferred to medium containing 5-BU (●) or ^{13}C-glucose-$^{15}NH_4Cl$ (△). Samples were taken at intervals and analyzed in the analytical ultracentrifuge by isopycnic centrifugation in CsCl. A similar experiment was performed with a culture growing in Casamino Acids-supplemented M9 which was transferred to a similar medium containing 5-BU instead of thymine (○). Per cent DNA replicated was calculated from the ultraviolet adsorption of the hybrid- and light-density peaks as the per cent DNA replicated = the per cent hybrid DNA/(200 minus per cent hybrid DNA)*

nella and also suggested that 5-BU does not alter this sequence. Cultures of 1660H were grown in supplemented M9 medium and were labeled for 8 min with ^{14}C-thymine. The culture was then grown in nonradioactive medium for 16 min and was finally labeled for another 8 min with ^{3}H-thymine. After this second radioactive pulse, the culture was either transferred to 5-BU medium or to $^{13}C^{15}N$ medium or was grown in nonradioactive thymine medium for another 75 min and then transferred to 5-BU medium. Samples were taken at intervals after transfer to the density labeling media and were analyzed by isopycnic centrifugation in CsCl (Fig. 7). In both thymine-$^{13}C^{15}N$ medium and in BU medium, replication of the ^{14}C-labeled DNA preceded the ^{3}H-labeled DNA. The sequential replication of the labels could also be observed after a generation of normal replication had intervened between the period of radioactive labeling and incorporation of the density label. Differences between the density-labeling experiments with 5-BU and $^{13}C^{15}N$ were consistent with the previously noted differences between DNA replication in thymine and 5-BU media (i.e., the longer lag before the appearance of hybrid DNA in 5-BU medium and the slower rate of replication in 5-BU medium). These differences could be eliminated by examining individual samples in the analytical centrifuge to determine the total amount of DNA replicated. When this was done and the results in Fig. 7 were graphed in a manner similar to Fig. 6, the $^{13}C^{15}N$ and 5-BU data were found to be almost the same (Fig. 8). It seemed clear that 5-BU does not disturb the sequence of replication. This was also shown clearly by another experiment. A culture growing in Casamino Acids-supplemented M9 was labeled with ^{14}C-thymine for 5 min and then was grown either in nonradioactive thymine medium or in 5-BU medium for 12 min. After this, the two cultures were each labeled with ^{3}H-thymine for 5 min, transferred to 5-BU medium, and sampled at intervals to determine the density of the ^{3}H- or ^{14}C-labeled DNA. The results in Fig. 9 clearly demonstrate that the ^{3}H- and ^{14}C-thymine label replicated in the same sequence whether the intervening period of growth occurred in 5-BU

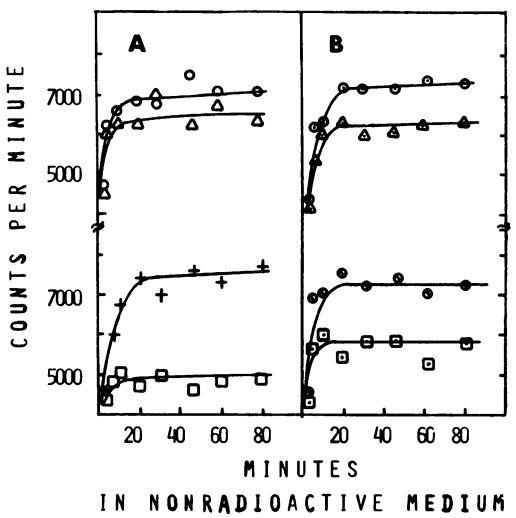

FIG. 5. *Residual incorporation after exposure to a pulse of radioactive thymine. Exponential cultures in Casamino Acids-supplemented M9 (A) or in M9 (B) were allowed to incorporate ^{3}H-thymine (10 μc per 2 μg per ml) for 5 or 8 min, respectively. The labeled cultures were then quickly (within 1 min) transferred to different nonradioactive media, and the incorporation of radioactivity into cold trichloroacetic acid-insoluble material was measured. Samples (0.2 ml) were taken. Symbols: (○, ⊙) media containing 20 μg of thymine per ml; (△, ⊿) media containing 2 μg of thymine per ml; (+, ⊘) media containing 20 μg of 5-BU per ml instead of thymine (also supplemented with deoxyguanosine; (□, ⊡) media containing thymine (20 μg/ml) but none of the required amino acids.*

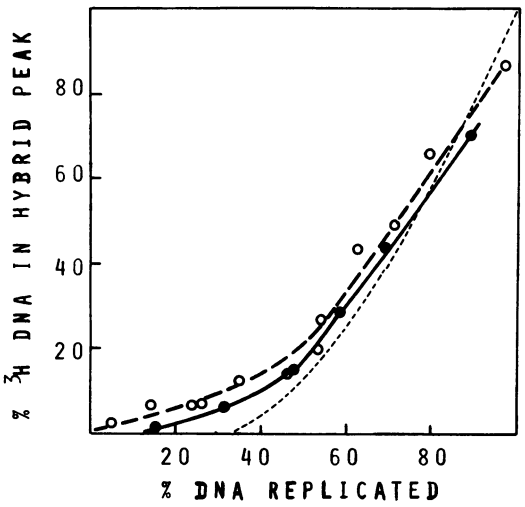

FIG. 6. *Sequential replication of Salmonella DNA I. Cultures of 1660H in M9 (●) or Casamino Acids-supplemented M9 (○) were grown for more than three generations in ^{14}C-thymine (0.5 μc per 2 μg per ml). When the cell titer reached 10^8 cells/ml, the cultures were labeled with ^{3}H-thymine (1.5 μc per 2 μg per ml) for one-eighth of their generation period and then were transferred to medium containing 5-BU instead of thymine. Seven 10-ml samples were taken; the DNA was extracted and analyzed by isopycnic centrifugation in CsCl. The per cent DNA replicated was obtained as the per cent of ^{14}C-DNA in the hybrid peak. Replication of E. coli 15T⁻ DNA (11) is also shown (dashed line).*

or in thymine. Thus, 5-BU did not appear to induce either a premature or a new replication sequence in *Salmonella*.

Amino acid starvation and chromosome replication. In *E. coli* and *B. subtilis*, amino acid starvation allows chromosome replication to be completed but prevents the initiation of a new cycle of replication (2, 8; R. Bird and K. G. Lark, *in press*; B. Wolf et al., Cold Spring Harbor Symp. Quant. Biol., *in press*). Thus, after such starvation, replication is initiated from a unique and heritable position on the chromosome.

When strain 1660H was starved for amino acids, DNA synthesis continued for a short time and then ceased. An increase in DNA per cell of about 50 to 60% was observed (H. K. Chan, Ph.D. Thesis, Kansas State Univ., Manhattan, 1968). This increase could be measured by the diphenlyamine reaction, incorporation of radioactive thymine, or the conversion of DNA to a hybrid density by incorporation of 5-BU. As in *E. coli*, when amino acids were again added, there was a resumption of DNA synthesis after a lag of about 30 min (Fig. 10). This was true in both M9 cultures and in Casamino Acids-supplemented M9 cultures (the results from an experiment in M9 medium are shown). As with *E. coli* (8), the lag in synthesis could be avoided by preventing DNA synthesis during the first 20 min after restoration of amino acids.

The slow initial rate of DNA synthesis was due to a low rate of DNA synthesis in individual cells (H. K. Chan, Ph.D. Thesis, Kansas State Univ., Manhattan, 1968) and to the fact that many of the cells did not resume replication until after a 20- to 30-min lag (Table 2).

If amino acid starvation blocks replication at a unique region of the chromosome, we may expect such a treatment to disturb the pattern of replication observed in Fig. 6. This was indeed, the case. Figure 11 presents the results of an experiment in which cultures were allowed to incorporate a pulse of ^{3}H-thymine after which they were starved for amino acids. Amino acids were eventually restored, together with 5-BU, and the pattern of replication was followed. A comparison with Fig. 5 makes it clear that the sequential pattern of replication was disrupted. Instead, the pulse-labeled DNA appeared to be replicated at random, as if either the replication sequence was disrupted or different cells were replicating the pulse at different times. The latter would be expected if all cells initiated replication from the same region of their chromosomes but contained the radioactive label in different regions of their chromosomes (as is the case when an exponential culture is pulse-labeled).

It is possible to demonstrate that amino acid starvation does not disrupt the sequential pattern of replication. A culture was starved for amino acids and divided into two portions. One was labeled with ^{3}H-thymine toward the end of the starvation period. The other was labeled with a pulse of ^{3}H-thymine immediately after starvation, i.e., upon restoration of amino acids. Both were subsequently transferred to 5-BU medium and replication was followed. Both labels were replicated as pulses and their replication was observed in the proper sequence (Fig. 12).

It was possible to demonstrate the presence of a unique and heritable region of the chromosome from which replication proceeds after amino acid starvation. This was done with the protocol shown in Table 3. In this type of experiment, a radioactive label is introduced into the chromosome after amino acid starvation and then attempts are made to relocate it after a second period of amino acid starvation. If, after starvation, replication proceeds from such a unique region, we expect the label to be replicated immediately after the second starvation, that is,

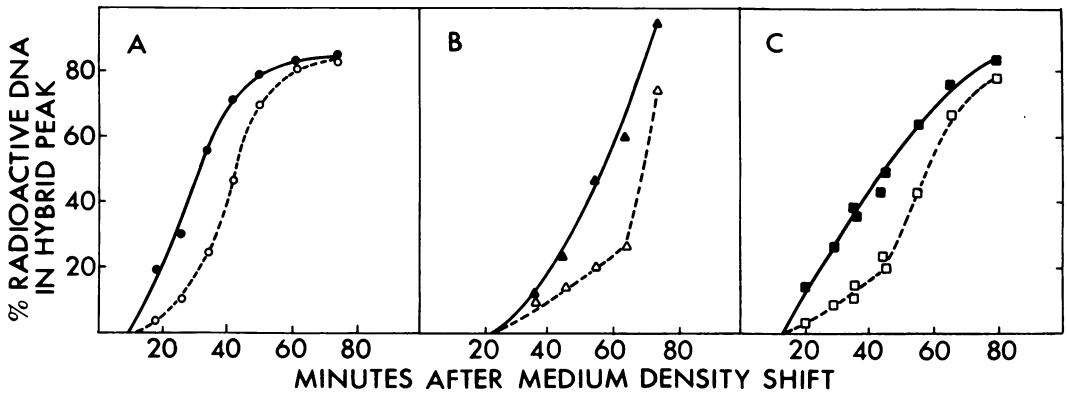

FIG. 7. *Sequential replication of Salmonella DNA II. Exponential cultures of 1660H (between 10^8 and 2 × 10^8 cells/ml) growing in M9 medium were labeled with ^{14}C-thymine (0.75 μc per 2 μg per ml) for 8 min. They were then washed and grown in nonradioactive medium for 16 min. The cultures were then incubated with ^3H-thymine (7.5 μc per 2 μg per ml) for 8 min and transferred to: (A) ^{13}C-glucose-$^{15}NH_4Cl$ M9 medium; (B) 5-BU medium; (C) M9 medium; after 75 min of growth they were transferred into 5-BU medium. Samples were taken after transfer into 5-BU or ^{13}C-^{15}N media and were analyzed as in the experiments in Fig. 6. Symbols: (●, ▲, ■), ^{14}C; (○, △, □), ^3H.*

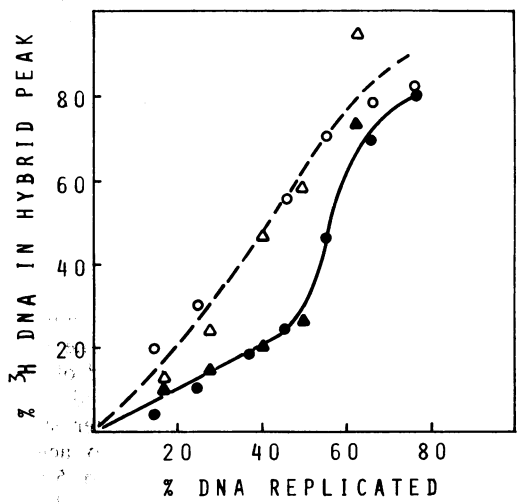

FIG. 8. *Effect of 5-BU incorporation on the sequential process of chromosome replication I. The data in Fig. 7 are compared on a graph of the type used in Fig. 6. The per cent DNA replication was obtained from measurement made in the analytical centrifuge. The ultraviolet adsorption of the hybrid- and light-density DNA peaks were compared as the per cent DNA replicated = the per cent hybrid DNA/(200-per cent hybrid DNA). Symbols: ○, ^{14}C replicated in 5-BU; △, ^{14}C replicated in ^{13}C-^{15}N; ●, ^3H replicated in 5-BU; ▲, ^3H replicated in ^{13}C-^{15}N.*

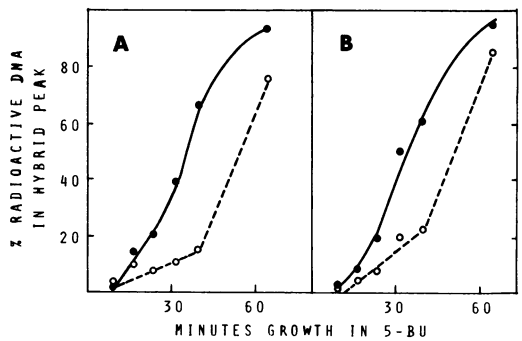

FIG. 9. *Effect of 5-BU incorporation on the sequential process of chromosome replication II. An exponential culture of 1660H (between 10^8 and 2 × 10^8 cells/ml) in Casamino Acids-supplemented M9 was labeled for 5 min with ^{14}C-thymine (0.2 μc per 2 μg per ml) and was divided into two portions. (A) Incubated in nonradioactive thymine medium for 12 min, then labeled with ^3H-thymine (1.5 μc per 2 μg per ml) for 5 min, and finally transferred to 5-BU medium. (B) Labeled as culture (A) but incubated in 5-BU medium for 12 min between the ^{14}C- and ^3H-labeling periods. After transfer to 5-BU, samples from both cultures were taken at the times shown and analyzed by isopycnic centrifugation in CsCl. Symbols: ●, ^{14}C-DNA; ○, ^3H-DNA.*

before the remainder of the chromosome. The results in Fig. 13A show that this was, indeed, the case. Such relocation of the label and its preferential replication was found in three different *Salmonella* strains. In each case, the generation period was about 40 min. In a control in which the second starvation treatment was omitted, preferential replication was not observed.

At slower growth rates, however, we could not relocate the radioactive labels (Fig. 13B). This was not a consequence of the absence of Casa-

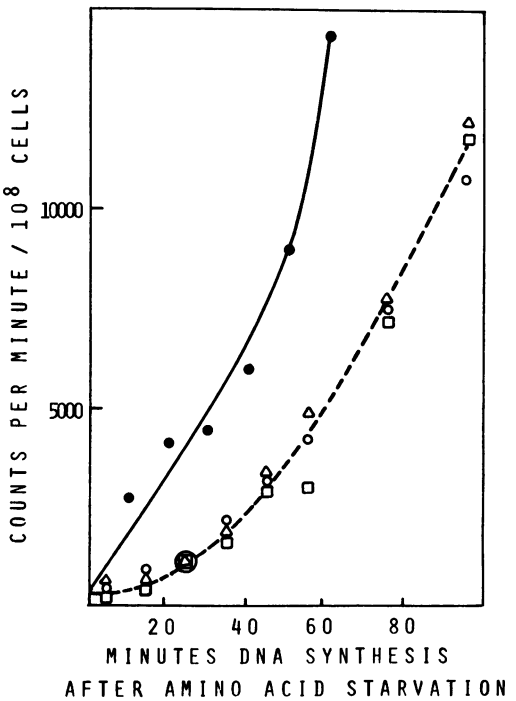

FIG. 10. *DNA synthesis after amino acid starvation of S. typhimurium. An exponential culture of 1660H (10^8 to 2 × 10^8 cells/ml) in M9 medium was starved for all required amino acids. After 80 (△), 100 (□), or 120 (○) min of starvation, amino acids and ^{3}H-thymine (10 μc per 2 μg per ml) were added. Incorporation of ^{3}H-thymine into 0.2-ml samples was measured. Another sample (●) was starved for amino acids for 100 min after which amino acids were restored but thymine was removed. ^{3}H-thymine (10 μc per 2 μg per ml) was added after 20 min and incorporation was measured.*

mino Acids since two of the strains, 3632 and 3660, were grown in the presence of Casamino Acids.

We had observed (Table 2) that cells which were growing slowly exhibited a greater heterogeneity in reinitiating replication after amino acid starvation than those from cultures with rapid growth (8; H. K. Chan, Ph.D. Thesis Kansas State Univ., Manhattan, 1968). Such heterogeneity could account for the observations in Fig. 13B.

To avoid this difficulty, a new technique was devised. The protocol for this technique is given in Table 4. After starvation for amino acids, we labeled cells during the first 25 min after readdition of amino acids. The culture was then starved again for amino acids. Under these conditions, only those cells which had incorporated ^{3}H-thymine and had initiated DNA synthesis could continue chromosome replication. After replication had proceeded, in the absence of amino acids,

TABLE 2. *Reinitiation of DNA synthesis after amino acid starvation*[a]

Time (min) after restoration of amino acids to the starved culture	Per cent of labeled cells	
	M9	M9 + Casamino Acids
20	69	86
30	82	88
40	84	90
50	88	92
60	95	93

[a] Exponential cultures (10^8 cells/ml) of 1660H in M9 or M9 + Casamino Acids were starved for all amino acids for 100 min. Amino acids were restored and 0.5-ml samples were taken at intervals and incubated for one-eighth of a generation period (5 or 8 min) with ^{3}H-thymine (100 μc per 2 μg per ml). The cells were then washed in the presence of nonradioactive thymine (1 μg/ml) and autoradiographs were prepared. After 16 days of exposure, these were developed and the per cent of labeled cells was scored. For each sample, 300 cells were counted. Most of the radioactive cells produced more than 10 silver grains in the emulsion.

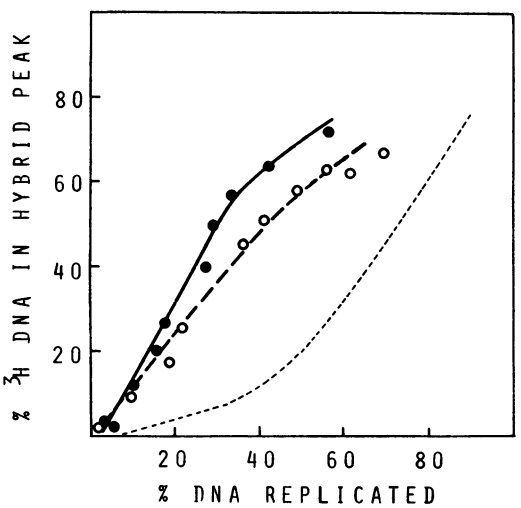

FIG. 11. *Replication of S. typhimurium DNA after amino acid starvation. Cultures of 1660H in M9 (●) and Casamino Acids-supplemented M9 (○) were treated as in the experiment in Fig. 6, except that they were starved for all essential amino acids, i.e., methionine, arginine, and tryptophan, between the period during which they incorporated ^{3}H-thymine and their subsequent growth in 5-BU medium. Analysis of the samples taken was carried out as in that experiment. Replication without an intervening period of amino acid starvation is also shown (dashed line).*

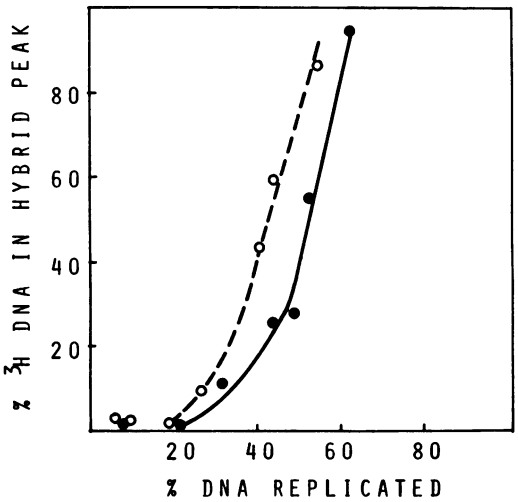

FIG. 12. *Replication of DNA labeled during or after amino acid starvation. A culture of 1660H (between 10^8 and 2 × 10^8 cells/ml) in Casamino Acids-supplemented M9 medium was starved for all amino acids. After 55 min of starvation, the culture was divided. One portion (○) was labeled with ³H-thymine (1.5 μc per 2 μg per ml) during 45 min of additional amino acid starvation. Another (●) was starved for 45 more min in nonradioactive medium and then, when amino acids were restored, was labeled with³H-thymine (2 μc per 2 μg per ml) for 25 min. After radioactive labeling, both samples were transferred to M9 medium containing 5-BU supplemented with Casamino Acids. Samples (10 ml) were taken at intervals and DNA replication was analyzed by isopycnic centrifugation in CsCl. Samples were analyzed in the analytical ultracentrifuge, and the per cent DNA replicated was calculated from the ultraviolet absorption of the light- and hybrid-density DNA peaks according to the following equation: per cent DNA replicated = per cent hybrid DNA/(200-per cent hybrid DNA).*

¹⁴C-thymine was added to label the portion of the chromosome immediately before replication ceased. Thus, if amino acid starvation blocks replication at a unique point of the chromosome, the region before this point would be labeled with ¹⁴C, whereas the region after this point would be labeled with ³H-thymine. Both radioactive labels should have been inserted into the same chromosome. After the second starvation period, replication was reinitiated by the addition of amino acids in the presence of 5-BU, and replication of the ¹⁴C- and ³H-labeled DNA was measured.

The ¹⁴C label and ³H label were inserted into unique locations in the chromosome (Fig. 14). The ³H-labeled DNA was replicated first and then the ¹⁴C-labeled DNA was replicated. Delay between the two is not surprising when one considers the interval between the administration of the two labels. A more revealing result was obtained when we did not reinitiate replication in 5-BU after the second amino acid starvation. Instead, the cultures were allowed to grow in thymine until the ³H-DNA had been replicated. Only then were the cells transferred to 5-BU medium. Under these conditions (Fig. 15), the ¹⁴C-labeled DNA and the ³H-labeled DNA were replicated together as would be expected if the two labels were inserted next to each other in the chromosome (Fig. 16). These results demonstrated that amino acid starvation blocks replication at a unique portion of the chromosome.

In the experiments in Fig. 14, the period between the replication of ³H-DNA and ¹⁴C-DNA represents the amount of DNA synthesized during the time which elapsed between exposure to the two labels. If DNA were synthesized at a slower rate, more time should be required to traverse the same portion of the chromosome. To test such a situation, we labeled a Casamino Acids-supplemented M9 culture (generation time, 42 min) according to the protocol in Table

TABLE 3. *Protocol for the experiments in Fig. 13*

Step	Treatment	Intended purpose
1	Amino acid starvation for 100 min	Alignment of chromosome at terminus
2	Addition of amino acids and tritiated thymine for 25 min	Reinitiation of chromosome replication at the origin and labeling of the beginning portion of the chromosome
3	Three generations of growth in ¹⁴C-thymine medium	Randomization of replicative process and uniform labeling of the chromosome
4	Amino acid starvation for 100 min	Alignment of chromosome at terminus
5	Transfer of culture to complete 5-BU medium	Reinitiation of chromosome replication at the same origin

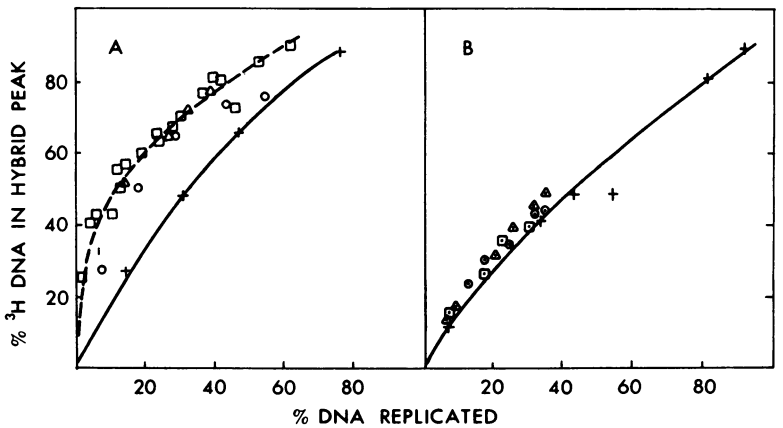

FIG. 13. *Relocation of radioactive DNA after amino acid starvation. Rapidly (A) or slowly (B) growing exponential cultures of E. coli were grown to a titer of 10^8 cells/ml and starved for all of the essential amino acids which they required (grown in M9 supplemented only with thymine). After 100 min, amino acids were restored together with ^{3}H-thymine (15 μc per 2 μg per ml). After incorporation of radioactive label for 25 min (A) or 28 min (B), the cultures were diluted eightfold, transferred to complete medium containing ^{14}C-thymine (0.05 μc per 2 μg per ml), and grown for three generations. The cultures were again starved for amino acids in M9 containing ^{14}C-thymine (0.05 μc per 2 μg per ml) for 100 min. Finally, they were transferred to 5-BU amino acid-containing medium and samples were taken at intervals for analysis as in the experiments in Fig. 6. The replication patterns are presented. □, 1660H grown in Casamino Acids-M9 medium; △, 3618 grown in Casamino acids-M9 medium; ○, 3612 grown in Casamino Acids-M9 medium; ▲, 3660 grown in Casamino Acids-M9 medium; ⊡, 3632 grown in Casamino Acids-M9 medium ○, 1660H grown in M9 medium; +, control experiments with 1660H grown in Casamino Acids-supplemented M9 medium (A) or in M9 medium (B) in which the second period of amino acid starvation was omitted. Thus, no attempt was made to relocate the label before transfer from ^{14}C-thymine into 5-BU.*

TABLE 4. *Protocol for the experiments in Fig. 14*

Step	Treatment	Intended Purpose
1	Amino acid starvation for 100 min	Alignment of chromosome at terminus
2	Addition of amino acids and tritiated thymine for 25 min	Initiation of chromosome replication and labeling of the beginning portion of the chromosome with tritium label
3	Amino acid starvation for 100 min	Alignment of chromosome at the terminus
	Addition of ^{14}C-thymine after 40 min of starvation	Labeling the terminal portion of the chromosome
4	Transfer of culture to complete 5-BU medium	Reinitiation of chromosome replication at the origin

4. The culture was then transferred to 5-BU-M9 medium (generation time, 65 min or longer), and amino acids were added to reinitiate replication. As can be seen in Fig. 17, 70 min elapsed between replication of the two labels, although the period between their replication in Casamino Acids medium was only 45 min (Fig. 14). This would indicate that the rate of DNA polymerization was reduced when the culture was transferred from Casamino Acids-supplemented to nonsupplemented medium.

DISCUSSION

As with *E. coli* 15T$^-$ (8), replication of the *Salmonella* chromosome is sequential and originates from a defined locus after starvation for amino acids. Incorporation of 5-BU into the *S. typhimurium* chromosome does not appear to alter the normal replication sequence (Fig. 7–9) or the sequence of replication which follows amino acid starvation (Fig. 12); nor does this analogue induce extra cycles of replication.

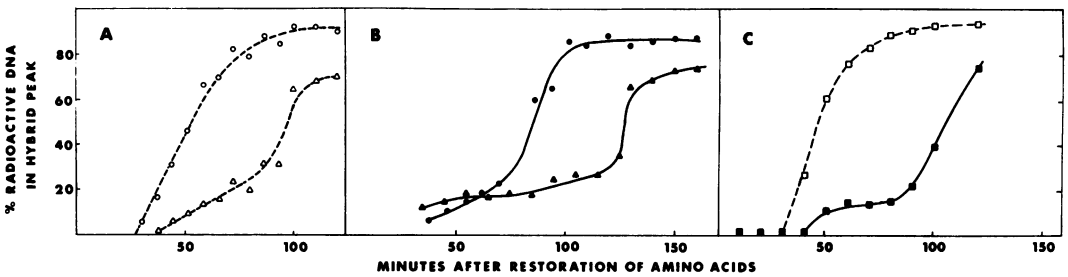

FIG. 14. *Cyclic sequential replication of the beginning and the terminal portion of the Salmonella or Escherichia chromosome I. Exponential cultures of S. typhimurium 1660H in Casamino Acids-supplemented M9 medium (A) and in M9 medium (B) and exponential cultures of E. coli 15T⁻ in M9 medium (C; each between 10^8 and 2×10^8 cells/ml) were starved for all amino acids for 100 min. Amino acids were then restored together with ^{3}H-thymine (2 μc per 2 μg per ml). After a 25- (A and C) or 28-min (B) period of radioactive labeling, the cultures were again starved for all amino acids by transferring them to nonradioactive M9 medium. After 40 min of starvation, ^{14}C-thymine (0.1 μc per 2 μg per ml) was added and starvation was continued for another 60 minutes. The doubly labeled bacteria were finally transferred to nonradioactive 5-BU medium, and samples were removed at intervals for analysis by isopycnic CsCl centrifugation. Symbols: (○, ●, □), ^{3}H; (△, ▲, ■), ^{14}C.*

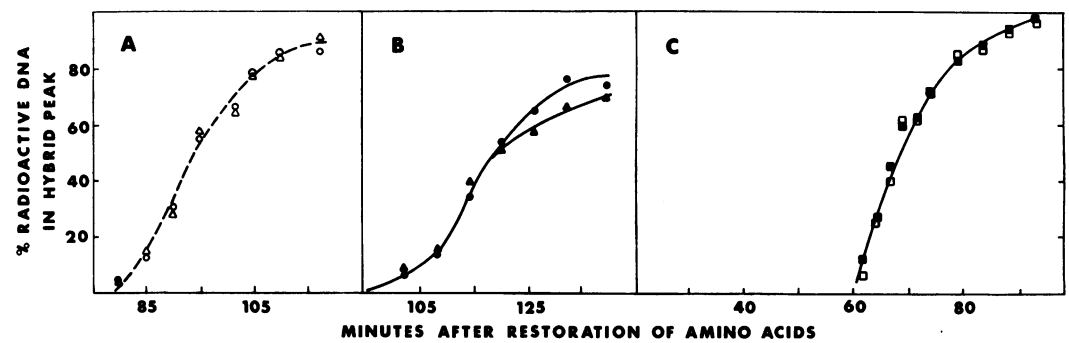

FIG. 15. *Cyclic sequential replication of the beginning and the terminal portion of the Salmonella or Escherichia chromosome II. Doubly labeled cells from the experiment in Fig. 14 were grown in nonradioactive thymine medium after the second amino acid starvation but prior to transfer to 5-BU medium. (A) S. typhimurium 1660H in Casamino Acids-M9 medium grown for 75 min in nonradioactive thymine medium before transfer to 5-BU medium. (B) S. typhimurium 1660H in M9 medium grown for 95 min in nonradioactive thymine medium before transfer to 5-BU medium. (C) E. coli 15T⁻ in M9 medium grown for 70 min in nonradioactive thymine medium before transfer to 5-BU medium. Samples were taken at intervals for analysis by isopycnic centrifugation in CsCl. Symbols: ○, ●, □, ^{3}H; △, ▲, ■, ^{14}C.*

The experiments described here utilized techniques previously applied to the study of chromosome replication in *E. coli* 15T⁻.

The results of these experiments were not as clear-cut as those previously obtained with *E. coli*. In part, this was due to the continued incorporation of ^{3}H-thymine after the transfer of cells to nonradioactive medium and to the delay in incorporating 5-BU or $^{13}C^{15}N$ when these density labels were introduced into the medium. These effects are most easily explained by assuming the presence of a large intracellular pool of thymine and other nucleotides. Such a pool has been observed in *E. coli* K-12 by L. Caro (*personal communication*). A more dramatic effect is the different result observed when slowly, instead of rapidly, growing cells were starved of amino acids (Fig. 13). Such a difference is most easily explained by assuming that slowly growing cells are more heterogeneous in reinitiating replication after amino acid starvation (Table 2). A similar effect has been observed in *E. coli* (11).

To avoid this difficulty, experiments were devised in which both the origin and terminus were labeled in all of those chromosomes which had initiated replication immediately after amino acid starvation. Such experiments (Fig. 14 and 15) demonstrated that, in both rapidly and slowly growing cultures, replication is initiated after amino acid starvation from a unique region of the chromosome and continues in a cyclic manner.

By labeling the chromosome origin and terminus, it is possible to obtain information about the replication cycle at fast and slow growth

rates. The data in Fig. 2 indicate that slowly growing cells contain fewer replication forks than cells growing rapidly. No period without replication (gap) was observed; i.e., all of the cells were labeled after exposure to a pulse of radioactive thymine. However, the length of the pulse, together with the period during which incorporation continued after removal of label (Fig. 5), could obscure a gap of as large as 20 min. On the other hand, it is clear from the experiments on sequential replication (Fig. 7) that an interval of 10 to 16 min can be detected between successive incorporation of a ^{3}H and a ^{14}C label. The fact that such an interval was not observed between the end of one cycle and the beginning of the next (Fig. 15) is strong evidence that there is little or no gap in replication. This observation also excludes a replication mechanism (13) in which each cell contains two chromosomes which are replicated alternately and in succession (since we have already pointed out that the labeling procedure insures incorporation of both the ^{3}H and ^{14}C labels into the same chromosome).

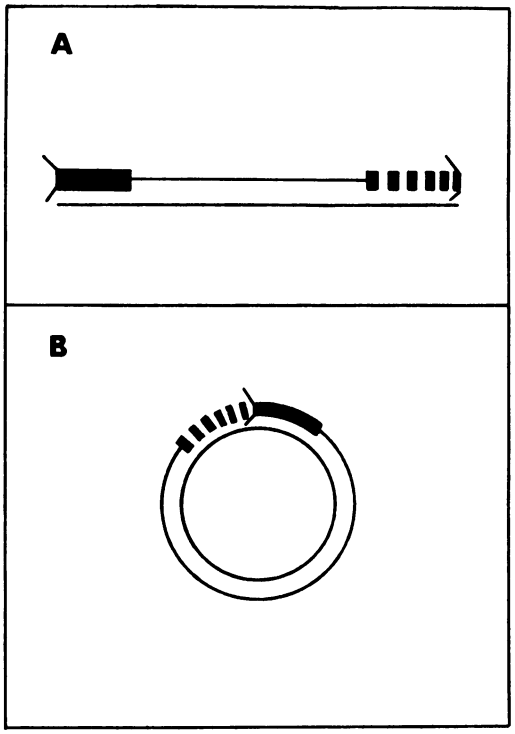

FIG. 16. *Position of ^{3}H (▬) and ^{14}C (■ ■ ■) labels inserted into the chromosome in the experiments in Fig. 14 and 15. (A) Possible linear arrangement; (B) circularization of A to indicate proximity of the two labels.*

Our data, however, can be explained by a model (5) in which new replication cycles are initiated early in the division cycle at rapid growth rates, but late in the division cycle for slow growth rates. Thus, at the rapid growth rate, each cell would contain, on the average, two replicating chromosomes in contrast to one at the slow growth rate. However, we also observed a decrease in the rate of chain elongation as the growth rate decreased without an observable cessation of the replication process (gap).

This decrease in chain elongation rate became directly observable when a rapidly growing culture was labeled at its origin and terminus while growing rapidly, after which replication was followed at a slower growth rate. Under these conditions, replication of the two labels was separated by a longer period at the slow growth rate (Fig. 17) than at the fast one (Fig. 14). The ratio

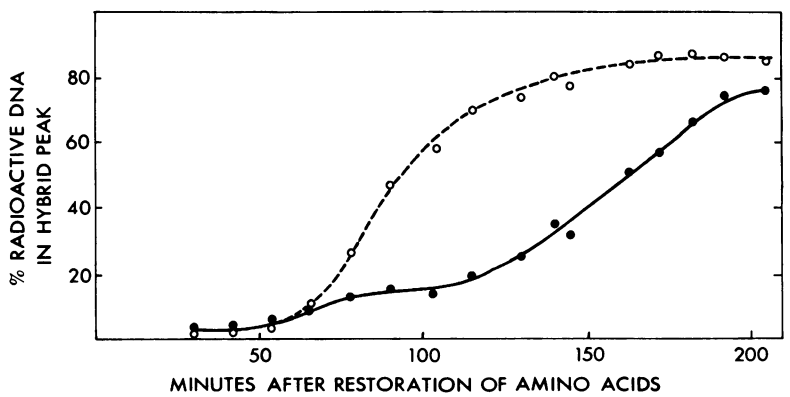

FIG. 17. *Replication of the beginning and the terminal portion of the Salmonella chromosome after removal of Casamino Acids as a supplement to M9 medium. A 1660H culture was labeled as in the experiment in Fig. 14A. However, after the second starvation, the cells were resuspended in M9 medium containing 5-BU which was not supplemented with Casamino Acids. (Although the three required amino acids were present). Samples were taken and analyzed as in the experiments in Fig. 14 and 15. Symbols: ○, ^{3}H; ●, ^{14}C.*

of the intervals corresponded, approximately, to the ratio of the two generation periods.

A recent extension of these experiments to *E. coli* 15T$^-$ has yielded similar conclusions for the rate of DNA synthesis in that organism (4), confirming earlier conclusions from autoradiographic data (10).

The recent work of Manor and Stent (J. Mol. Biol., *in press*) indicates that the rate of ribonucleic acid (RNA) chain elongation in *E. coli* also decreases with the growth rate. Perhaps the changes in rate of RNA and DNA polymerization reflect the scarcity of a common precursor required for the synthesis of these polynucleotides, or the need for a unique condition of the DNA template used in their biosynthesis.

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