*REPLICATION OF THE ESCHERICHIA COLI K12 CHROMOSOME**

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Evidence now available indicates that the *Escherichia coli* chromosome is a single, circular DNA molecule whose replication proceeds in a semiconservative manner, at a single point, and unidirectionally.[1, 2] However, it is still unclear whether in the *E. coli* chromosome, replication starts at a fixed point and proceeds in a fixed order. There exist already several genetic investigations and theories aimed at resolving these questions pertaining to the replication of the *E. coli* chromosome,[3–5] but since the conclusions drawn from these works are, in part, contradictory, further investigations are evidently needed.

It has been shown[6] that a transducing particle of phage P1 grown on *thy*$^-$ bacteria previously grown in a medium containing bromouracil (BU) carries DNA that is a fragment of the BU-labeled bacterial chromosome which already existed at the time of the infection. Based on this observation, the following experiment seemed to offer an approach to the investigation of the dynamics of replication of the *E. coli* chromosome. The growth of bacteria is synchronized by bringing their DNA replication cycles to completion, after which the cells are transferred to a medium containing bromouracil and allowed to grow for various lengths of time before infection with P1. Phage growth and lysis take place in a medium without bromouracil. If one centrifuges this P1 lysate in a CsCl density gradient and assays the transducing activities, one should be able to determine the order of the replication of the chromosome from the distribution of transducing particles carrying various loci. In fact, we found an ordered replication of the *E. coli* chromosome with these experiments. During the course of the investigation, however, we encountered an unexpected effect on chromosome replication caused by the addition of bromouracil to the medium. Detailed investigations of the replication of the chromosome in the presence of bromouracil are reported below.

Materials and Methods.—Bacterial and phage strains: A virulent mutant of phage P1kc[6] was used and is referred to as P1 in this paper. Bacterial strains used were derivatives of *E. coli* K12: strains HfrC *thy*$^-$ *met*$^-$, HfrH *thy*$^-$, and F$^-$W3110 *thy*$^-$ are thymineless derivatives of Hfr Cavalli,[7] Hfr Hayes,[7] and W3110 (Lederberg), respectively, and used as donors. AB440 *arg*$^-$ *mtl*$^-$ *xyl*$^-$ *his*$^-$ *gal*$^-$ *lac*$^-$ *pro*$^-$ *thr*$^-$ *leu*$^-$ (Adelberg), JE3423 *arg*$^-$ *lys*$^-$ (Hirota), W4183 *argG*$^-$ (Udaka), and X36 *arg*$^-$ *ara*$^-$ *lac*$^-$ *gal*$^-$ *pyrF*$^-$ *try*$^-$ *his*$^-$ *purC*$^-$ *thy*$^-$ *Str*^{r} *mal*$^-$ *xyl*$^-$ *mtl*$^-$ *T1*^{r} (Wolf) are recipients in transduction. AB440 was also used as an indicator for P1 plaque assay. *E. coli* 15T$^-$ *arg*$^-$ *met*$^-$ *try*$^-$ *thy*$^-$ (Lark) was used for comparison purposes in some experiments.

Media: The medium used for growth of bacteria was tris Casamino acids glucose medium (TCG)[8] or TG medium (Difco Casamino acids in TCG medium was replaced by 1.1 gm/liter of NH_4Cl) supplemented with 2 μg/ml of thymine or 5 μg/ml of 5-bromouracil (BU medium) and 10 μg/ml of required amino acids. The doubling time of these bacteria in TCG and TG media supplemented with thymine was about 40 and 50 min, respectively. L-broth, L-agar, soft agar, and minimal agar[6] were used for transduction experiments. Dilution buffer contains 10^{-2} *M* tris buffer, pH 7.4, 5 gm/liter of NaCl and 10 mg/liter of bovine serum albumin.

Labeling and density gradient centrifugation of DNA: The labeling of total cellular DNA was accomplished by growing bacteria for several generations in a medium containing $P^{32}O_4$ (0.01 μc/μg P) or C^{14}-thymine (0.01 μc/μg thymine). A portion of the growing chromosome was labeled by a pulse of H^3-thymine (0.5 μc/μg thymine) followed by a chase with 200 μg/ml of thymine

for 1 min. The culture was filtered on a Millipore membrane filter, washed, and suspended in a medium with $P^{32}O_4$ or C^{14}-thymine and without H^3-thymine. Aliquots of the culture were taken at intervals into tubes containing crushed ice. DNA was extracted as described elsewhere,[9] except that the phenol extraction was done by hand shaking. The average size of the DNA thus gm/ extracted was about one fiftieth of a whole bacterial chromosome. The DNA was mixed with CsCl solution at a final density of 1.725 gm cm^{-3}, centrifuged in an SW39 rotor of a Spinco ultracentrifuge at 35,000 rpm for 48 hr at 15°C. After the run, 60–65 fractions were obtained by collecting drops after puncturing the bottom of the centrifuge tube. The radioactivities of the DNA fractions of each sample were measured in a scintillation spectrometer.

Synchronization of DNA replication: Amino acid starvation method: The method described by Lark[10] was generally followed. Phenethyl alcohol treatment:[11] To an exponentially growing culture of bacteria in TCG medium, phenethyl alcohol (Wako Pure Chemical Industry) was added to a final concentration of 0.3%. After incubation for 2 hr the culture was filtered and the cells were washed and suspended in a fresh medium. DNA synthesis then resumed after a lag of 15–20 min.

Preparation of P1 lysate and density gradient centrifugation of P1: An exponential culture of bacteria in TCG medium containing thymine was transferred to BU medium; samples were withdrawn after intervals of growth in BU medium and given KCN (2×10^{-3} *M*), $CaCl_2$ (2.5×10^{-3} *M*), and P1 at a multiplicity of infection of about three phages per cell. After 20 min the infected cells were collected by centrifugation and suspended in a medium containing thymine. The suspension was incubated for 90 min with shaking until lysis and sterilized with chloroform. The lysate was mixed to give 3.2 ml of CsCl solution of density 1.472 gm cm^{-3} which contains 0.5% Casamino acids and 0.01 *M* tris buffer, pH 7.5. The mixture was centrifuged in an SW39 rotor for 18 hr at 23,000 rpm at 15°C. After centrifugation about 60 fractions were obtained by drop collection. Each fraction was diluted in a suitable amount of dilution buffer and examined for infectivity and transducing activity.

Transduction: Recipient bacteria were grown in L-broth to a concentration of about 5×10^8 cells/ml, collected by centrifugation, and suspended in TG medium containing 2.5×10^{-3} *M* $CaCl_2$ to give 5×10^8 cells/ml. The bacteria were infected with the phage in an equal portion of each fraction except those around the peak of infective particles, which were diluted to give a multiplicity of infection of less than 0.1, and incubated for 30 min at 37°C. The cells were spread on minimal agar plates containing appropriate supplements for selection of transductants and anti-P1 serum to K = 0.2/plate to reduce interference by phage produced on the plates. (We are indebted to Dr. Hisao Uchida for suggesting this procedure.) Colonies were counted after incubation at 37°C for 48 hr.

Results.—Alteration of the replication point of the K12 chromosome by the addition of bromouracil: An exponential culture of W3110 *thy*$^-$ in TCG medium containing thymine and $P^{32}O_4$ was pulse-labeled with H^3-thymine for six minutes (about one eighth of a generation), and transferred to BU medium. Samples of the culture were taken at intervals during growth in the medium. DNA was extracted from the cells and analyzed for the distribution of radioactivity in a CsCl density gradient. If bromouracil is incorporated in continuation of the previous growing point of DNA, the pulse-labeled portion of the DNA should be found in association with BU-labeled DNA only after a majority of the total DNA has already replicated. This is the case in strain 15T$^-$, as was shown by Lark,[10] and can also be seen in Figure 1*b*. Evidently here the bulk of the total DNA was transferred to the hybrid density band before any similar transfer of the H^3-labeled region. In contrast, in strain W3110 *thy*$^-$ replication of the pulse-labeled DNA occurred soon after the transfer of the culture to BU medium (Fig. 1*a*), and proceeded with almost the same rate as that of the total DNA (Fig. 2) until about 80 per cent of total H^3-DNA was replicated. The rate of H^3-DNA replication was then reduced. Fully heavy DNA appeared when about 70 per cent of DNA initially present had replicated.

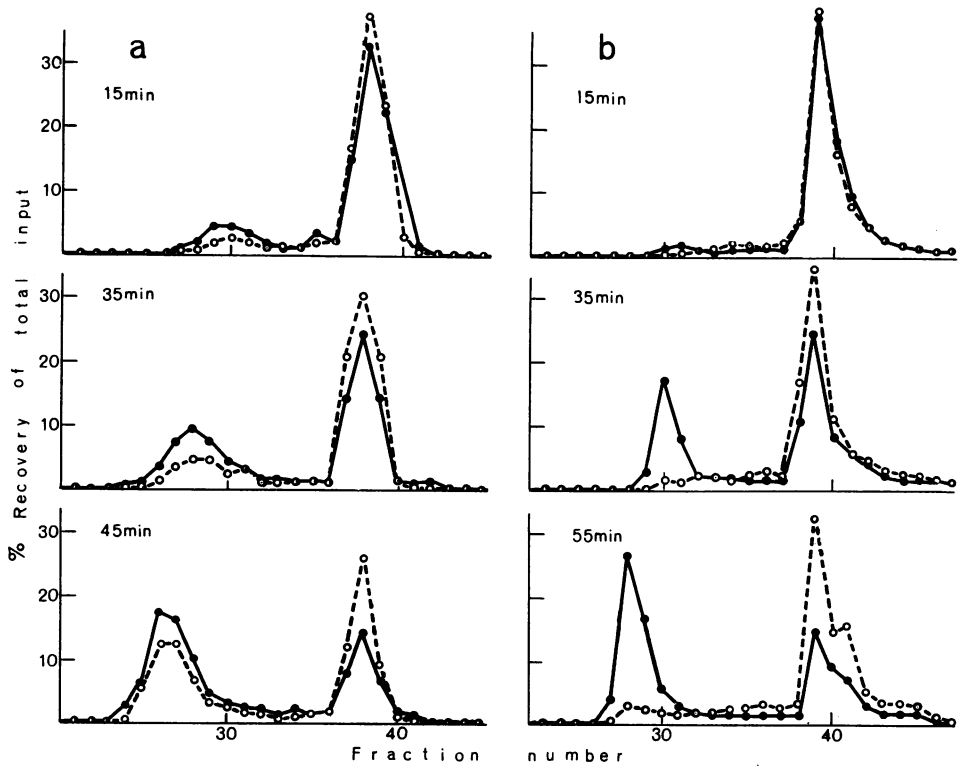

FIG. 1.—Distribution of radioactivity in CsCl density gradients of doubly labeled DNA. An exponential culture labeled continuously by P^{32} was pulse labeled by H^3-thymine and transferred to BU medium containing $P^{32}O_4$. DNA was extracted at the times indicated, and centrifuged. (*a*) F⁻W3110 *thy*⁻, (*b*) 15T⁻; ● for P^{32}-total DNA, ○ for H^3-pulse-labeled DNA.

Similar results were obtained with HfrC *thy*⁻ *met*⁻ and HfrH *thy*⁻. The nearly proportional replication of the labeled region with the total DNA clearly indicates that in these K12 strains the replication sequence of the chromosome is altered by transferring cells to BU medium.

Initiation point induced by the addition of bromouracil: It has been shown that the replication of the chromosome of 15T⁻ is synchronized by amino acid starvation[10] or phenethyl alcohol treatment[11, 12] to completion of a cycle and starts at a fixed point after resumption of protein synthesis. In the following this point will be called the *origin* of the chromosome. It should be possible, by synchronizing the growth of the chromosome before the addition of bromouracil to locate the starting point of the replication induced by bromouracil. If the initiation point of the replication induced by the addition of bromouracil is the same as the chromosomal *origin*, DNA near the *origin* should replicate upon transferring the cells to BU medium when only a small part of the total DNA has replicated.

Cells of HfrC *thy*⁻ *met*⁻ grown in TG medium containing methionine and C^{14}-thymine were starved of methionine for two hours during which the total DNA increased about 50 per cent. The culture was transferred to a complete medium and H^3-thymine was added to label the region adjacent to the *origin*. H^3-thymine was

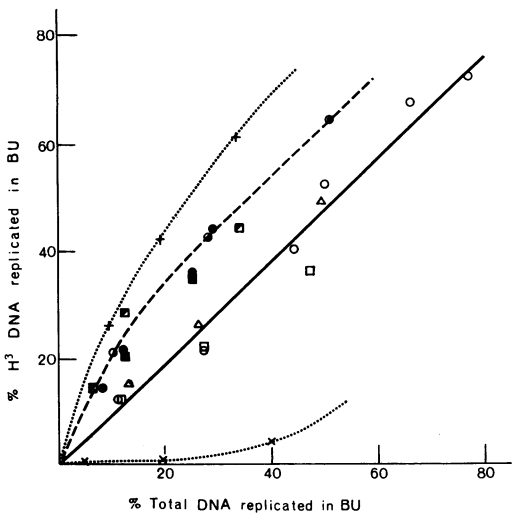

FIG. 2.—Pattern of replication of pulse-labeled DNA. (*i*) DNA was pulse labeled by H^3-thymine during exponential growth and then grown in BU medium (△ F⁻W3110 *thy*⁻, □ HfrC *thy*⁻, ○ HfrH *thy*⁻, × 15T⁻). (*ii*) DNA was pulse labeled after synchronization by amino acid starvation (■ HfrC *thy*⁻) or phenethyl alcohol treatment (◩ HfrC *thy*⁻, ◑ HfrH *thy*⁻), and grown in BU medium. In another experiment, the growth was randomized after synchronization and pulse labeling with H^3-thymine. The culture was then transferred to BU medium (● HfrH *thy*⁻). (*iii*) DNA was labeled by H^3-thymine after thymine starvation. The culture was randomized and transferred to BU medium (⊙ HfrH *thy*⁻). (*iv*) DNA of 15T⁻ was labeled by H^3-thymine after the first amino acid starvation. Following randomization of growth and a second amino acid starvation, the culture was transferred to BU medium. (+ 15T⁻).

removed by filtration after 30 minutes (a lag and the initial replication period), and the cells were resuspended in a medium containing methionine and bromouracil. In other experiments, the cells were diluted tenfold in a medium with methionine and thymine and allowed to grow until the cell number had increased tenfold prior to the addition of bromouracil. By that time the synchronized growth was randomized. The DNA samples from the cells taken at intervals during the growth in BU medium were analyzed for the density distribution of the radioactivity. Experiments were also carried out in which cultures of HfrC *thy*⁻ *met*⁻ and Hfr*Hthy*⁻ were treated with phenethyl alcohol. The patterns of replication of H^3-labeled DNA are shown in Figure 2. In every case a large fraction of H^3-labeled DNA has moved to the hybrid density position early after the transfer of the cells to BU medium. This may mean that the initiation of the replication in BU medium occurred from the *origin* of the chromosome. Additional evidence which supports this interpretation was obtained from an experiment in which the *origin* was pulse labeled after thymine starvation for 30 minutes, and the replication of the pulse-labeled region was followed in BU medium. The result is presented in Figure 2. Again pulse-labeled DNA replicated early during the growth in BU medium.

In Figure 2, the result of an experiment performed with 15T⁻ is also shown. DNA of 15T⁻ cells was pulse-labeled after an amino acid starvation and their growth was randomized in a complete medium. After a second starvation bromouracil was added. If the amino acid starvation synchronizes the chromosomal replication perfectly, and if replication proceeds synchronously in BU medium, a strict preferential replication of H^3-labeled region of 15T⁻ DNA should occur at a very early time. In accord with Lark's observation,[13] a large portion of H^3-DNA replicated early in the growth in BU medium (Fig. 2). The observed rate of the initial replication of H^3-DNA in 15T⁻ DNA was lower than expected, probably because neither one of the required conditions was exactly satisfied. This may also be true or more pronounced for the K12 strains. Furthermore, the synthesis

of DNA may occur at the original growing point in some small proportion of K12 cells which would reduce the initial rate of replication of the pulse-labeled region. Under these circumstances, we consider that the similarity in the initial rate of replication of the H^3-labeled region of 15T$^-$ and K12 strains can be taken as a strong indication that the point at which chromosome replication is initiated by the addition of bromouracil is the chromosomal *origin* or the region very close to it.

Genetic localization of the initiation point induced by bromouracil and the order of replication of chromosomal markers: The alteration of the replication sequence by bromouracil and the probable identity of the chromosomal *origin* and the initiation point induced by bromouracil were revealed by density gradient analysis of DNA. With this in mind we attempted to locate genetically the initiation point and the order of the replication of the K12 chromosome by transduction analysis.

P1 phage lysates were periodically prepared from an exponential culture of HfrH *thy*$^-$ after a shift to BU medium, as described in *Materials and Methods*. The lysates were centrifuged in CsCl density gradients and the fractions were examined for the transducing activities of the markers shown in Figure 3. The patterns of distribution of transducing particles observed for each of these markers are presented in Figure 4. Experiments of the same type were carried out with HfrC *thy*$^-$ *met*$^-$ and F$^-$W3110 *thy*$^-$, and typical results are summarized in Table 1 where the percentage of the transducing activity of each marker in the hybrid fraction (replicated) to the total activity is shown. The results clearly indicate that all markers do not have an equal probablity of replicating upon transfer to BU medium, but that there is a preferred region of early replication as was shown by the density gradient analyses of DNA discussed above. The results appear to suggest that the region at which the chromosomal replication commences is located between the *lysine* and *histidine* markers and proceeds in a clockwise fashion regardless of the sexual state of the cell.

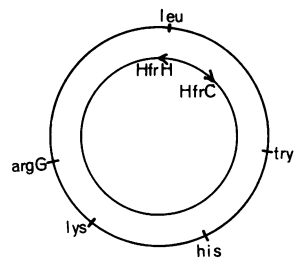

FIG. 3.—The genetic map of *E. coli* K12 chromosome, showing the location of markers used.

The results, however, show some inconsistency which is not in full accord with this interpretation. Several causes of the inconsistency in the order and the rate of the replication of the markers should be discussed. First, transducing particles of intermediate density between hybrid and light may be produced, especially at a very early period of growth after the transfer to BU medium. The markers replicated earlier would, therefore, overlap the light region more. As is clearly seen in the density-gradient analysis of the DNA (Fig. 1), DNA which was synthesized soon after the transfer was lighter than that synthesized later. Lags in the replacement of thymine by bromouracil causes underestimation of transducing activity of markers replicated early. Second, a variation may exist among cells in the duration of the lag before the starting of replication as well as in the rate of synthesis of DNA in BU medium. In accord with this assumption, twice-duplicated DNA was found in the fully heavy region, while some DNA (approximately 30%) was not yet replicated. This effect of bromouracil addition must decrease synchronous replication of the markers. An additional complication is the *leucine*

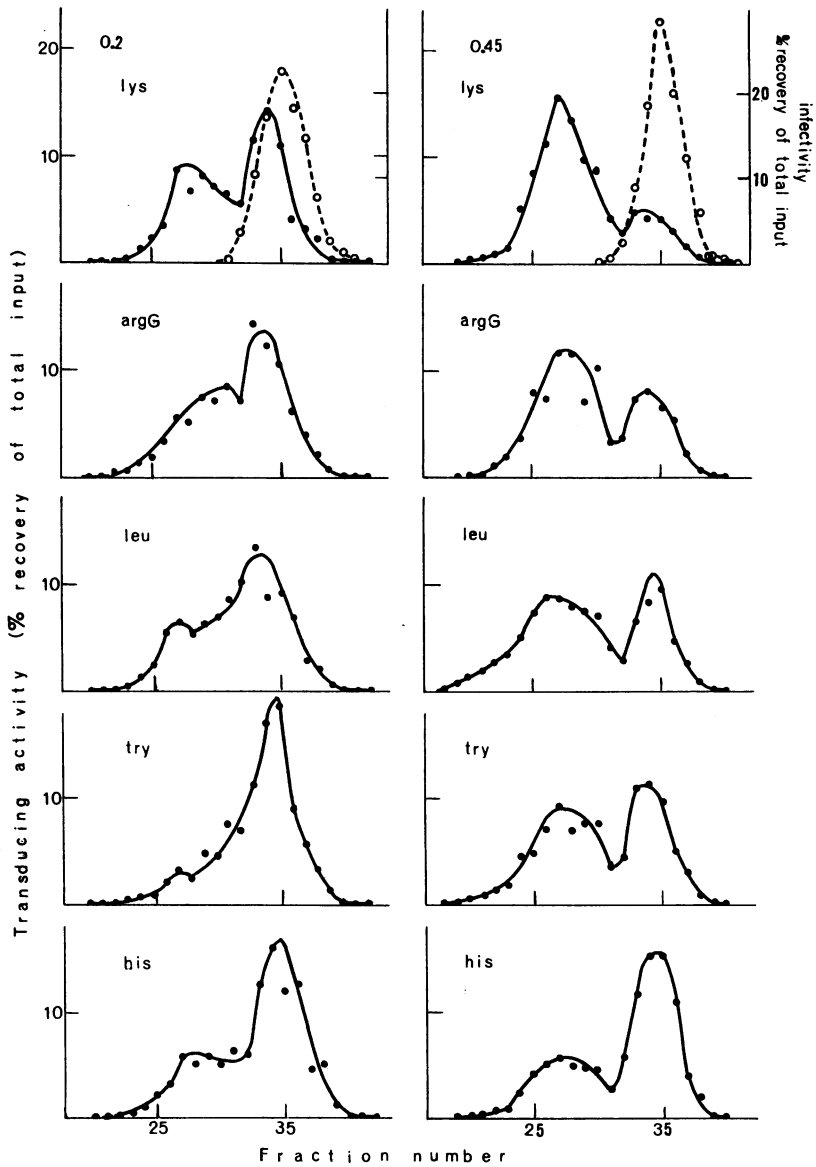

FIG. 4.—Density analyses of the transducing activities of P1 lysates from an exponential culture of HfrH *thy*⁻ partially labeled by bromouracil. ● for transducing activity, ○ for infectivity. Numbers in the top frames show the fraction of DNA replicated in BU medium when the cells were infected with P1.

marker which was the first marker to appear in the fully heavy region. The *threonine* and *arabinose* markers were also found in fully heavy fractions when *leucine* was there, but other markers shown in the table were not. This was observed in the experiments with F⁻W3110 and HfrC *thy*⁻ *met*⁻ indicating that it has no correlation with the sexual state of the cells. The fraction of the activity of *leucine* found in the fully heavy region was greater than the fraction of the

TABLE 1

PERCENTAGE OF TRANSDUCING ACTIVITY OF THE REPLICATED MARKERS TO TOTAL ACTIVITY

Strains	Fraction of DNA replicated in BU medium*	Markers				
		lys	*argG*	*leu*	*try*	*his*
F⁻W3110 *thy*⁻	0.2	17	31	17	18	17
	0.45	34	75	—	25	—
	0.8	91	74	54	—	38
HfrC *thy*⁻ *met*⁻	0.15	21	13	15	8	4
	0.4	—	28	21	—	8
	0.6	70	48	59	33	21
	0.8	88	76	62†	50	23
HfrH *thy*	0.2	35	36	25	14	8
	0.45	63	47	36	38	12

The calculations were done as follows: (1) The heavier half of the light particle region is overlapped with the hybrid particles. The activity of the light particle region was calculated from the lighter half of the light particle region, according to the observation that light transducing particles show the same distribution in CsCl density gradient as infective particles and have a density 0.002 heavier than the infective particles.[6] (2) The activity of the hybrid region is the difference between the total activity and the light activity. Assuming that P1 can pick up both of the hybrid strands of a chromosome replicating in BU medium, the activity of the hybrid region was divided by 2, to obtain the fractional activity of the replicated marker in BU medium.

* The fraction of DNA replicated in BU medium was obtained from the density analysis of DNA totally labeled by $P^{32}O_4$.

† 35% of the *leucine* activity was found in the fully heavy region. None of the other markers was found in this region.

activity of *histidine* in the hybrid region. This may suggest that fully heavy markers were formed before the completion of one cycle of growth of chromosomes in BU medium. It must be noted, however, that at an early time the markers were in the proposed order.

Discussion.—In 15T⁻, as Lark has shown,[10] bromouracil can be incorporated into DNA strands to continue the previous replication sequence. In contrast, it has been shown in this report that bromouracil does change the pattern of the replication of the growing chromosome in strains of K12. Several models for the altered replication by bromouracil have been constructed and the feasibility of explaining our results by these models will now be considered: (1) The initiation of the replication at random point(s) on a chromosome. This model predicts that the label at the *origin* replicates proportionately to total DNA replication, and the density distribution of the transducing particles of every marker would be equal. (2) The reversal of direction after completion of a replication cycle. According to this model, fully heavy material should appear very early after transfer to a BU medium. (3) The replication proceeds from both the *origin* and the replication point which previously existed. This condition would also cause an early appearance of fully heavy material after the transfer of cells to the BU medium. (4) The replication is initiated at only one of the two *origins* of a partially replicated chromosome. With this mechanism, only half of the DNA pulse-labeled during random growth would be replicated. Our observations are not in accord with the predictions made by any of these models. Instead we propose that the replication is initiated prematurely at both of the *origins* of a replicating chromosome and that the replication at the growing point existing prior to the addition of bromouracil ceases. This model is different from the pattern of premature initiation induced by thymine starvation in 15T⁻ which was reported to occur both at the replication point previously existing and at one *origin* of two partial replicas of a replicating chromosome.[13] The possibility that the initiation occurs at one *origin* at first and

then another *origin* starts to replicate after the completion of the half replica of a replicating chromosome is, although unlikely, not completely eliminated.

The replication sequence induced by bromouracil was genetically analyzed according to the above model. The results clearly showed an order of replication of the markers and revealed the presence of a genetically defined initiation point and orientation of the replication of a chromosome. The early appearance of the *leucine* marker in the fully heavy region cannot be explained at the present time. There may possibly be another effect of bromouracil which appears when the incubation in BU medium lasts a long time.

From these observations and considerations we tend to favor the conclusion that an initiation point and an order of replication of the chromosome were revealed by the present experiments. Namely, the initiation point is located somewhere between the *lysine* and *histidine* markers, and the replication proceeds clockwise, regardless of the sexual state of the cell.

The existence of a definite origin on the genetic map has been revealed in another laboratory.[14] The location and the direction of replication are in general agreement with the above conclusion.

Summary.—It was shown that in *E. coli* K12, the sequence of chromosome replication was altered by growing the cells in a medium containing bromouracil instead of thymine. In experiments with double labeling of DNA, the initiation point of the replication reoriented by bromouracil was shown to be the same as the point where the replication initiates after the amino acid starvation or phenethyl alcohol treatment.

By the density analysis of P1 transducing particles, the initiation point was located between the *lysine* and *histidine* markers, and the order of replication was shown to be clockwise, regardless of the sexual state of the cells.

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