DISTINCTION BETWEEN EARLY AND LATE GENES REPLICATED IN *SALMONELLA TYPHIMURIUM*, AS DETERMINED BY NITROSOGUANIDINE MUTAGENESIS

by

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INTRODUCTION

Replication of chromosomes is a fundamental event in bacteria that is geared to cell division, highly regulated by an unknown mechanism. Jacob et al. (1963) postulated a "replicon model" in which an "initiator" interacts with the "replicator", the fixed genetic site of replication origin, to initiate the synthesis of a new chromosome, which then runs to completion along a circular DNA structure. They also predicted the association of the chromosome with the cell membrane. Cairns (1963 a, b), via radioautography, showed that the Escherichia coli chromosome is circular, and postulated that replication starts from a fixed origin and proceeds in a uni-direction. Many workers have been trying to determine the genetic site of origin and direction of replication, including Nagata (1962, 1963) who first reported that in E. coli Hfr strains the integration site of F-factor is the replication origin, but in F^- strains the origin is variable. Most of these studies have been with E. coli or Bacillus subtilis.

The purpose of this investigation was to identify the genetic region of origin and the direction of chromosome replication of Salmonella typhimurium LT2 F^- which is phylogenetically related to E. coli.

Cérda-Olmedo et al. (1968) treated synchronized cells of E. coli at various times with nitrosoguanidine (NG), a mutagen known to act preferentially on the replication point of the chromosome, and scored the number of revertants of various mutant markers scattered around the chromosome. By this method they found the relative order of gene replication starting from the origin. The following studies are based on their assumption.

Basically two experimental systems were used. One was the comparison of marker revertibility between log phase cells and stationary phase cells, and the

other was the sequential mutagenesis as described by Cérda-Olmedo et al. (1968). In the former experiment, the cells at log phase and stationary phase were both treated with NG and a comparison was made of the number of revertants between them (ratio of stationary/log). Since there is a preference for early genes when stationary cultures are mutagenized (Botstein et al., 1968), it should be possible to utilize these ratios in distinguishing early and late genes in the replication cycle. An early marker should have a higher stationary/log ratio than a marker that is replicated late. The validity of this experiment will be discussed comparing the results with those obtained by the sequential mutagenesis experiments.

LITERATURE REVIEW

The Bacterial Chromosome

An important discovery in considering the control mechanism of replication is that the bacterial chromosome consists of one continuous DNA molecule with circular likage groups (Jacob and Wollman, 1961).

Cairns (1963 a, b) showed directly by radioautography that the E. coli chromosome is a closed, circular structure. This circular DNA was calculated to be 1,100μ long, and the molecular weight to be approximately 2.8×10^9 dalton, coinciding with the molecular weight calculated from the amount of DNA per nucleus. The size of a silver grain in radioautography is about 1μ, corresponding to 4,000 nucleotides, but the fine structure between grains may account for additional nucleotides. These radioautographs permit an estimate of 10^7 nucleotide pairs per chromosome, which is in good agreement with chemical analysis. Berns and Thomas (1965) extracted Hemophilus influenzae DNA by very mild method and found 2.5 per cent (weight percentage) amino acids still contained in DNA after the purification. So it is not excluded that some linkers are involved in the integrity of the DNA molecule.

In other bacteria, such as S. typhimurium and B. subtilis, genetic analysis also revealed a single linkage group with circular structure. But no direct evidence of a circular structure has been reported. Nevertheless, it may be generalized that the bacterial chromosome consists of a single, circular DNA molecule.

Sequential Replication

Meselson and Stahl (1958) provided the evidence that the bacterial chromosome replicates in a sequential as well as semiconservative manner. In their experi-

ments, E. coli grown in heavy N^{15}-labeled medium for a number of generations was transfered into light N^{14}-medium. DNA was extracted at intervals after transfer and centrifuged in cesium chloride gradient. By this method they found that all of the N^{15}-DNA banded in the position with hybrid density (one strand labeled with N^{15} and the other with N^{14}) by the end of one cycle of replication. These results indicated that DNA replication occurs in a semiconservative and sequential manner.

Radioautography of the E. coli chromosome showed the structure of replicating form and its sequential replication (Cairns, 1963 a, b). Lark et al. (1963) also demonstrated that replication proceeds sequentially from the chromosome origin. They pulse-labeled E. coli $15T^-$ with H^3-thymine and then transferred cells into 5-bromouracil (BU) medium. DNA was extracted at various times after transfer. The radioactive DNA did not replicate until all of the DNA had replicated.

In other experiments, a steady exponential culture of E. coli K12 was pulse-labeled with H^3-thymidine and allowed to grow in a heavy medium containing C^{13}-glucose and $N^{15}H_4Cl$ (Nagata and Meselson, 1968). The distribution of radioactivity appeared periodically at one generation intervals, elegantly demonstrating the existence of a fixed origin and sequential replication.

Using similar techniques in S. typhimurium the sequential replication was also shown by Chan and Lark (1969).

The sequential replication and the fixed origin in the B. subtilis chromosome were determined by two different experiments: marker frequency analysis and density transfer experiment (Yoshikawa and Sueoka, 1963 a, Sueoka and Yoshikawa, 1963 b). In former experiment, they predicted that markers close to the replication origin should be more frequent than those close to the terminus. The frequen-

cies of transformation of early markers were twofold when compared to DNA from exponential phase cells with DNA extracted from stationary or spore cells. The culture in the latter experiment was grown in D_2O medium. The markers were transferred from heavy DNA to hybrid DNA in the same order as the former experiment.

Replication Forks

The considerable variations of the growth rate of bacteria were observed by changing the types of media. Schaechter et al. (1959), using radioautography, showed that DNA replication in glucose medium occupies over 90 per cent of the generation time (about 40 min) both in S. typhimurium and E. coli. Similar work was done by Lark (1966) using E. coli. She showed that in rapidly growing cells DNA replication occupies more than 90 per cent of a generation time but in slowly growing cells replication occupies only a fraction of a generation time. Clark and Maaløe (1967), using synchronized cultures of E. coli B/r, measured the increase of H^3-thymidine incorporation after adding chloramphenicol (CM). They found that the increase of incorporation occurs about 20 min prior to division at which a new round of DNA replication is initiated. Helmstetter and his collaborators (Helmstetter, 1967, Helmstetter and Cooper, 1968, Helmstetter et al., 1968) have determined the rate of DNA synthesis during the division cycle of E. coli B/r. The exponentially growing cells were pulse-labeled with C^{14}-thymidine and filtered onto the membrane filters and eluted after the holder was inverted. Based on the marker frequency theory by Sueoka and Yoshikawa (1965), the cells in which DNA replication is initiated should have the highest radioactivity. Thus, they proposed a model in which the time required for the replication forks to traverse the chromosome is constant, about 40 min at 37°C and at rapid growth rates more than one replication point per chromosome exist.

At slow growth rates the travel time is increased and there is a gap between the termination of one round of replication and the initiation of the next round. Similar results were obtained by Bird and Lark (1968) using a different technique. Multiple replication forks were also observed in B. subtilis spores germinated in rich medium (Yoshikawa et al., 1964).

Role of Membrane in DNA Replication

Bacteria do not have the mitotic apparatus of higher organisms. In spite of that, extrachromosomal DNA replicates in a harmonious manner with chromosmal DNA. Jacob et al. (1963) proposed that F-factor and probably the other replicons are attached to the cell membrane, after studying the thermosensitive mutants of F-factor.

Ryter and Landman (1964) observed directly the association of the chromosome with a cellular organelle, called mesosome, by electron microscopy of B. subtilis. They also found that L-form and protoplasts of B. subtilis lose their mesosome and that the chromosome attaches directly to the cell membrane.

Studying the segregation pattern of the chromosomes by radioautography, Lark (1966) proposed a replication model, in which one strand of the chromosome is permanently attached to the cell membrane by protein, the replicator. The other is attached to the new site on the membrane via a protein, the proreplicator, by the interaction with initiator. Lark and his collaborators (Eberle and Lark, 1966, Chai and Lark, 1967) actually observed that DNA associates permanently with a part of the cell envelope which is synthesized during the generation after synthesis of the DNA itself. So they proposed the non-random distribution of sister chromatids into the daughter cells. In contrast with their conclusios, Ryter et al. (1968) indicated that the association between the bacterial nucleus and the membrane does not determine the distribution of the

Greenberg, 1965, Freese and Bautz-Freese, 1966), the isolated tobacco mosaic virus RNA and the polynuceotide used as a messenger RNA in vitro (Chandra et al., 1967).

The inactivation pattern may be somehow different from the mutagenesity pattern. Because NG induced few mutation in transforming DNA of B. subtilis (Freese and Bautz-Freese, 1966) and isolated TMV RNA (Signer and Fraenkel-Conrat, 1967). The mutagenesity of NG on nucleic acids is still under investigation. NG treatment of TMV RNA, and other RNAs in water modified extensively the guanidine residue and also the modification of a small fraction of adenine was found. In 67 per cent dimethylformamide the cytosine is altered. Considering the difference in response to NG treatment of naked RNA and RNA in the TMV rod, these authors concluded that the reaction of NG with RNA is conformation-dependent. Chandra et al. (1967) showed that all nucleic acids treated with NG were methylated. The methylation of bases were proved by using NG treated polynucleotides; U→C and A→C. This supports the transcriptional mutation, AT→GC, found in Neurospora crassa (Malling and DeSerres, 1967).

In S. typhimurium, NG and diethylsulfate have about the same range of mutagenesity when compared the mutation and reversion patterns (Eisenstark et al., 1965). Also comparing the reversions with NG and 2-aminopurine, which is able to induce both types of transional mutations; AT→GC or GC→AT (Freese, 1963). Eisenstark et al. (1965) found that NG could induce nontransitional, as well as transitional mutations, remaining the possibility that NG causes deletion mutation. Using the same species Whitfield et al. (1966 a, b) found that NG could induce missense and nonsense but not frameshift mutations.

In E. coli S, all mutations induced by the radiomimetic agents as well as UV in the lac region appear to be of the same type, only being different in their

old DNA.

The growing point of the chromosome as ewll as the initiation point, was shown to be attached to the cell membrane. Hanawalt and Ray (1964) suggested that in E. coli the nascent BU-labeled DNA is bound to protein. Ganesan and Lederberg (1965) reported that the fast-sedimenting fraction (DNA-membrane complex) in B. subtilis has up to 25 per cent of the total assayable DNA polymerase activity. It was also shown in B. subtilis that the replication point and the chromosome origin-terminus junction are attached to the membrane (Sueoka and Quinn, 1968). Ramareddy and Reiter (1970), using the same organism, showed that the fraction of DNA associated with the membrane increases when DNA is being synthesized but decreased upon halt of DNA synthesis. They also demonstrated that the association is only dependent on DNA synthesis.

Induction of Initiation

Protein synthesis is required for the initiation of DNA replication (Maaløe and Hanawalt, 1961, Lark et al., 1963, Lark and Lark, 1964). From the difference in effects between amino acid starvation and chloramphenicol (CM) treatment in E. coli, Lark and Lark (1964) found at least two proteins participated in the control of DNA synthesis. One is CM sensitive and the other is CM resistant. The latter could be a structural protein essential for the association with membrane. Lark and Renger (1969) suggested a third step required for the initiation of DNA synthesis by the fact that the initiation did not occur immediately after necessary protein synthesis was completed. This step could bethe configuration change in DNA molecule or cell morphology change during which the two daughter chromatids are segregated. They also showed that CM resistant step is not timed by DNA synthesis, agreed with the model proposed by Helmstetter et al. (1968). While Ward and Glaser (1969) looked at the timing of

H^3-thymidine uptake blocked by chloramphenicol in physiologically synchronized E. coli B/r culture and found that there are at least two separate processes which must be completed in order to initiate a new round of replication. Their observation is consistent with the first two steps postulated by Lark and Lark (1964) but they did not find any evidence for the third step which Lark and Renger (1969) postulated. The discrepancies may be due to the differences of strains or treatments such as amino acid starvation or thymine starvation.

DNA replication occurs synchronously in B. subtilis germinating spores. Using this system Yoshikawa (1965) determined the timing of CM inhibition on the protein synthesis, consequently DNA synthesis. He concluded that protein synthesis, possibly the inducible protein, is required immediately before the initiation of DNA replication.

Under abnormal conditions the initiation of DNA replication can be induced. If a culture is deprived of thymine, the number of survivors decreases in a logarithmic way. But if thymine is added back to the medium before thymine-less death occurs, DNA synthesis is initiated and the rate of DNA synthesis increases twice as much as before thymine is starved. Pritchard and Lark (1964) showed that in E. coli the replication is initiated prematurely at only one of the two partial replicas. The UV irradiation also causes the premature initiation (Hewitt and Billen, 1965). Abe and Tomizawa (1967) reported that BU causes the premature initiation at both of the origins of a replicating chromosome and that the replication stops at the growing point existing prior to the addition of BU. In any case, DNA replication is slowed down or stopped, while protein synthesis continues. Therefore, initiation occurs normally (Yoshikawa and Haas, 1968, Helmstetter et al., 1968), or possibly this treatment activates the structural genes for initiation and the initiator is induced.

DNA Replication Model

Since Cairns' direct observation of the replicating form of the E. coli chromosome, numerous models for DNA replication have been proposed. Among these is the "rolling circle model" elaborated by Gilbert and Dressler (1968) for phage production and the circularity of DNA. This model states that the positive strand is nicked by a specific endonuclease and the 5' end of this strand is attached to the membrane. The minus strand, always closed, serves as template for the chain elongation of the 3' end of the old positive strand. The new negative strand is also synthesized from the 5' to 3' using peeled off positive strand. DNA polymerase adds the nucleotides to the 3'-OH producing the short pieces that are to be joined by ligase. Thus, the long tail produced is cut into individual genomes that can be circularized. The torque on the circular molecule is imagined to unwind the DNA, requiring the closed circle molecule for DNA synthesis. Thus, initiation of replication cycle is induced asymmetrically. In E. coli, Bird and Lark (1968) found that the premature initiation which occurs during rapid growth involves both of the origins of the replicating chromosome. This finding ruled out the "rolling circle model" for E. coli, but this model could well explain phage DNA synthesis and particular cases such as premature initiation after thymine starvation (Pritchard and Lark, 1964) or the transfer of chromosome in conjugation.

Replication Origin-Terminus and Direction

As mentioned before experiments indicate that the bacterial chromosome has a fixed origin and that replication proceeds sequentially along the chromosome. But these physical studies neither identified the origin on the map nor determined the direction of replication. Nagata (1962, 1963 a) first visualized a genetic approach to determine the origin and direction of replication. He induced two

prophages λ and 424 by UV in synchronized E. coli Hfr and F⁻ strains. He found that the relative position of prophages on the chromosome map agrees with the relative time of replication in terms of per cent increase of DNA in Hfr strains but not in F⁻ strains. Therefore, he concluded that in Hfr strains the integration site of F-factor is the origin and that the replication proceeds toward the conjugational origin. On the other hand, in F⁻ strains no unique site of origin of replication was found, although the polarity of replication was observed.

In B. subtilis the replication origin was identified and also the replication direction was determined by Yoshikawa and Sueoka (1963 a) who predicted that marker frequency near the origin should be higher than that near the terminus in an exponentially growing population (this was theoretically shown later by them) (1965). The actual data obtained by the transformation technique agreed with the theory.

The timing of enzyme induction may be used by many workers to determine the origin and direction of bacterial chromosome replication. In this technique the frequency of genes transduced by phage was examined. Berg and Caro (1967) used P1 phage for transduction and examined the relative frequency of various markers. They found that in Hfr strains of E. coli the integration site of F-factor is not the replication origin. After amino acid starvation, the cells were transferred into the medium containing BU and were infected by P1 phage. The lysate was centrifuged in cesium chloride density gradient and BU containing phage particles were separated. These phages carrying various loci were assayed for transduction. In this way, Abe and Tomizawa (1967) found that the replication origin locates between lys and his genes and replication proceeds clockwise in both Hfr and F⁻ strains of E. coli. Using a similar technique Wolf et al. (1968 a, b) found that in some Hfr strains replication starts clockwise from

the origin between lys and xyl but another strain has its origin in the region between pro and gal with counterclockwise replication. Therefore, it was postulated that strain differences exist and also more than one site on the chromosome can be the initiation point of DNA replication.

Caro and Berg (1968, 1969) used two different systems of transduction. One system is similar to Abe and Tomizawa type using BU incorporated phage particle In another system, P1 lysate was made from the exponentially growing culture at various growth rates, and they analyzed the distribution of marker freqency. Both types of experiments showed that most strains of E. coli K12 have replication origin close to xyl gene and clockwise replication. They also reported evidence of bi-directional replication which may be caused by amino acid starvation or BU treatment.

In S. typhimurium Nishioka (1970) using a similar transduction technique via P22 phage containing BU, suggested that replication proceeds in both directions from the origin close to ilvA.

Altenbern (1966) introduced a new mapping technique using NG mutagenesis. He synchronized Staphylococcus aureus with phenethyl alcohol and treated cells with NG at various times, although he did not try to identify the replication origin of the chromosome, this method gave the basis for the sequential mutagenesis used later to identify the origin and direction of replication. Cérda-Olmedo and Hanawalt (1967) reported that NG mutagenizes preferentially at the replication point of chromosome. The cells were synchronized by amino acid starvation, followed by thymine starvation, and treated with NG at various times. The revertants were scored. A given type of mutant should have its maximum revertibility at the time when the corresponding gene is being replicated. Thus, Cérda-Olmedo et al. (1968) found that all strains tested (E. coli $15T^-$, K12 Hfr and K12 F^-) have a fixed origin near 50 min of the map, between arg and tyr

and clockwise direction of replication.

Both the transduction technique and sequential mutagenesis with NG have been used by additional workers (Wolf et al., 1968, Ward and Glaser, 1969). There are some discrepancies in the reports as to the origin and direction of chromosome replication, but it may be reconciled that under normal condtion the replication in E. coli may start from the region between 8 and 9 o'clock on the map and proceeds clockwise. The discrepancies may arise from the abnormal treatments.

Synchronization

It is very important to obtain synchronously growing cells since it allows the quantitative biochemical investigation on a large number of cells of the population reflecting a behavior of a single cell. Furthermore, it is more valuable if DNA replication is highly related to cell division, in other words, DNA replicates synchronously.

Synchronous division of bacteria was first induced by temperature shifts (Lark and Maaløe, 1956) or thymine starvation (Barner and Cohen, 1956). The synchronized culture obtained by these methods may not necessarily reflect normally dividing cells. To avoid possible artifacts, synchronization was induced by selecting the same age cells assuming that they have the same size (Yanagita and Maruyama, 1956) or they have the same density.

An ingenious method has been developed by Helmstetter and Cummings (1964), in which cells were attached to a membrane filter surface and eluted from it after inverting the filter. In this procedure only new daughter cells are released constantly from the membrane and these cells showed a good synchronization in their growth.

Also Cutler and Evans (1966) have establised another physiological synchro-

nization technique. They grew bacteria, E. coli and Proteus vulgaris, to the stationary phase and inoculated the portion at early stationary stage into fresh medium. Repeating this twice they obtained the synchronized culture at least for three generations. They also concluded from their preliminary results that genomic synchrony occurs by this technique.

Biological Effects of Nitrosoguanidine

Since Mandel and Greenberg (1960) first reported the mutagenesity of nitrosoguanidine, the wide investigation of biological effects has been performed. Its effects vary depending on the conditions applied such as temperature, pH, buffers, time of treatment, the organisms to be used. Although the molecular mechanism of the action is still obscure and some inconveniences exist in its application, NG is widely used for its extreme effectiveness as a mutagen.

i) Optimal Condition for Mutagenesis

As a matter of course, optimal condtions should be determined for adequate application of a given mutagen. Adelberg et al. (1965) first tried to do it using E. coli. They tested the survival and mutagenesis changing the functions, such as the growth phase of the treated cells, pH, treatment time, the medium, and NG concentration. The optimal conditions were obtained by taking cells from log phase, treatment with NG for 15 to 30 min at pH 6.0. Cérda-Olmedo et al. (1968), looking at the chemical properties of NG, also determined the optimum mode of application. They also found that NG induces more revertants in log phase culture than the culture in which replication is completed, and concluded that NG acts effectively on replication point of DNA. This idea opened up the technique to determine the DNA replication origin and its direction.

ii) Mutagenesity of Nitrosoguanidine

NG can inactivate transforming ability of B. subtilis DNA (Terawaki and

mutation producing efficiency (Zampieri and Greenberg, 1967). This nonspecific property may be due to the peculiarity of E. coli strain S or lac region tested.

Some disadvantages of NG as a mutagen were reported that NG induced the double mutants (Eisenstark et al., 1965, Adelberg et al., 1965) and some NG-induced mutants are highly instable (Eisenstark et al., 1965).

Cérda-Olmedo (1968), performing the systematic investigation of biological effects of NG, postulated that NG could cause misreading of the genetic code, when looked at NG action in streptomycin resistant cells.

In other bacteria NG causes supressor mutation, hence phenotypically revertants (Shizosaccharomyces pombe), and its action is the same as UV but not as nitrous acid or spontaneous mutations (Loprieno and Clarke, 1966).

iii) Addtional Effects of NG

NG is effective in inducing both chromosomal and nonchromosomal mutations in Chlamydomonas reinhardi. The agent caused nonchromosomal mutations to streptomycin resistance and dependence, to neamine resistance and dependence. Only streptomycin is known to induce nonchromosomal mutation in this species, but this mutagen does not induce chromosomal mutations (Sager, 1962).

Lingens and Oltmanns (1966) also reported extrachromosomal mutation in yeast, "petite" mitochondrial mutation. In Euglena chloroplast mutations were induced by NG with very little lethality (MacCalla, 1965).

In higher organisms chromosome aberrations by NG were reported in Vicia faba (Gichner et al., 1963), Arabidopsis taliana (Müller and Gichner, 1964), and Allium cepa (Sax and Sax, 1966).

MATERIALS AND METHODS

Materials

i) Bacterial Strains

S. typhimurium LT2 F^- derivatives were used in all of the experiments and are listed in Table 1. The good revertible mutants were selected by the revertibility test with NG from many mutants (Eisenstark et al., 1965)(Table 1).

ii) Media

Minimal medium was prepared by mixing equal amounts of 2X-minimal salts solution and distilled water supplemented with 1 per cent of volume of 40 per cent glucose. 2X-minimal salts solution contains the following salts in grams per liter: K_2HPO_4, 21; KH_2PO_4, 9; $(NH_4)_2SO_4$, 2; sodium citrate, 1; and $MgSO_4 \cdot 7H_2O$, 0.1.

Minimal agar was prepared in the same way as minimal medium, with 2X-minimal agar replaced for distilled water. 2X-minimal agar contains 18 grams of Ionagar in 1 liter of water.

Nutrient broth was made by mixing 8grams of dehydrated nutrient broth, and 5 grams of NaCl in 1 liter of water.

Nutrient agar was prepared by mixing 13.5grams of Ionagar, 8 grams of dehydrated nutrient broth and 5 grams of NaCl in 1 liter of water.

Minimal soft agar (MSA) was prepared by mixing 5 grams of NaCl and 6 grams of Ionagar in 1 liter of water.

iii) Chemicals

Nitrosoguanidine (N-methyl-N'-nitro-N-nitrosoguanidine), purchased from Aldrich Chemical Co., Milwaukee, was used at the concentration 0.1 mg/ml in tris maleate buffer at pH 5.5.

iv) Buffers

TABLE 1. Bacterial Strains used in Mutagenesis Experiments

Cistron	Allele #	KSU Strain #	Mutational origin
argE	13	5012	2-aminopurine
cysC	244	5604	2-aminopurine
	389	5739	X-rays
cysG	385	5735	X-rays
cysJ	299	5652	Spontaneous
his	2	6452	
ilvA	12	7159	Spontaneous
	17	7164	Spontaneous
lys	6	1619	
phe	30	8515	Spontaneous
purE	52	4601	Spontaneous
trpD	52	4601	Spontaneous
thy	66	2015	Nitrosoguanidine

Tris salts buffer was prepared by mixing 9 per cent solution A, 90 per cent distilled water and 1 per cent solution B in this order, and pH was adjusted to 7.4 with either NaOH or HCl and autoclaved. Solution A contains following components: tris(hydroxymethyl)-aminoethane, 120 grams dissolved in 500 milliliters of distilled water; concentrated HCl, 75 milliliters; KCl, 20 grams; NH_4Cl, 20 grams; Na_2HPO_4, 3.5 grams; and distilled water 400 milliliters. Solution B is composed of 5 grams of $MgCl_2 \cdot 6H_2O$ and 100 milliliters of distilled water.

Tris maleate buffer was prepared by mixing 50 milliliters of solution I, 13.1 milliliters of 0.2 molar NaOH and enough distilled water to complete 200 milliliters. pH was adjusted to 5.5. If necessary, the buffer was sterilized by filtration. Solution I contains 0.2 molar tris acid maleate (24.2g/1 tris-(hydroxymethyl)-aminoethane and 23.2g/1 maleic acid).

Methods

i) Cell Growth and Cell Number Count

Liquid cultures were grown at 37°C with aeration. An overnight culture was diluted into the fresh medium (usually 1:20 dilution) and growth was followed by optical density at 450mμ in a Klett-Summerson photoelectric colorimeter and actual cell number in a Coulter Counter Model F.

ii) Revertibility Test

Cells (ca. 5×10^8) were spread on minimal agar plate without the nutritional requirement under study. The satulated NG in tris maleate buffer was dropped to the surface of the plate and allowed to diffuse through the agar. After two days incubation at 37°C, in the good revertible mutants the colonies appeared in a zone where NG was in optimum concentration. These mutants were used for further mutagenesis stusy.

iii) Mutagenesis Assay

The mutagenized cells were plated onto the minimal agar plate without the requirement under study for the reversion of nutritional requirement. Colonies were counted after two days incubation at 37°C.

iv) Synchronization

For synchronization, the method of Cutler and Evans (1966) was basically followed. 5 ml of overnight culture was grown in the fresh medium (100ml) until the stationary phase. 5 ml of the stationary phase culture was removed at a critical time (generally 40-70 min after the culture has reached the stationary phase), and transferred into 100 ml of fresh medium without washing. After transfer, a portion of culture was removed at 10 min intervals. Optical density and cell number were measured by the methods mentioned above.

v) NG Mutagenesis for the Comparison of Revertibility at Stationary Phase and Log Phase

One mililiter of the samples were removed at 10 min intervals for 40 min from the log phase culture (ca. $2x10^8$) grown in the minimal medium supplemented with requirements. The samples were filtered through Millipore filter (0.45μ pore size), washed with tris salts buffer and resuspended into three mililiters of NG solution for 10 min without nutrients and aeration at 37°C. After washing cells with tris salts buffer to remove the extra NG, cells were spread onto minimal plate without the nutritional requirements under study. The number of cells added to the plate was adjusted according to the number of revertants anticipated, to permit accurate counting. For the control one mililiter of samples were taken, washed, resuspended into tris salts buffer and plated onto the same medium as used in NG treated cells. After two days incubation at 37°C, the revertants which appeared on the surface of plate were counted. The average of the number of colonies per plate was considered to indicate the revertibility

of that mutant at log phase after standardization. 50 min after the culture had reached the stationary phase (ca. 2×10^9) the samples were taken at 10 min intervals for 40 min and the same procedure was followed above.

vi) Sequential Mutagenesis

The exactly same procedure as mentioned in v) was followed using synchronized culture for two or three generations.

RESULTS

i) Revertibility Tests

Table 2 shows the results of revertibility tests. From this screening, mutants were selected that reveted with NG to an extent that would be useful in later quantitative experiments. If spontaneous revertants were too numerous in a particular strain, it was eliminated for future experiments.

ii) Synchronization

In accordance with the method of Cutler and Evans (1966), synchronized cells were obtained by taking samples from a culture that had just completed its log phase of growth. The arrows in Fig. 1 designate the two times that samples were removed; A at 10 to 20 min and B at 50 to 80 min after the culture reached stationary phase. These samples were then transferred to 100 ml of warmed fresh medium without washing (Fig. 1). As shown in the figure, the cells from the early stationary stage (A) did not show good synchrony, while the cells from the middle stationary phase (B) did grow in a moderately synchronized manner. Synchrony in Fig. 1 was measured in terms of the increase in cell number.

iii) Comparison of Revertibility of Cells at Stationary Phase and Log Phase

There is an evidence that NG mutagenizes bases at the replication point to a greater degree than the remainder of the DNA molecule (Cérda-Olmedo, et al., 1968). If this is the case, NG incorporated into cells at stationary phase (followed by subsequent growth in fresh medium), should act more effectively on early markers than on late markers. On the other hand, in a population of log phase cells, there is equal opportunity for NG mutagenesis for every gene in the cell. In such a case, it should be possible to identify early and late markers in the replication cycle by comparing the ratio of revertant numbers of stationary/log phase cells upon NG treatment. The following is a description of the experi-

TABLE 2. Revertibility Test

Cistron	Allele #	KSU Strain #	Revertibility
argB	75	5073	-
	165	5163	++ (sp)
argE	2	5001	++
	8	5007	-
	9	5008	++
	12	5011	-
	13	5012	++
aroB	3	5207	+++ (sp)
	4	5208	-
	99	5304	-
	127	5332	-
	130	5335	$+^{\pm}_{-}$
	161	5366	-
	165	5370	-
aroC	2	5206	-
	36	5241	-
cysC	7	5406	-
	43	5437	-
	78	5458	-
	88	5468	$\pm$
	117	5491	-
	119	5493	-
	120	5494	$\pm$

Cistron	Allele #	KSU Strain #	Revertibility
cysC	207	5576	-
	208	5577	$\pm$
	213	5582	$\pm$
	215	5584	$\pm$
	216	5585	$\pm$
	218	5587	+
	219	5588	++
	241	5601	-
	243	5603	-
	244	5604	++
	246	5606	-
	247	5607	-
	291	5645	-
	354	5706	$\pm$
	355	5707	-
	356	5708	-
	357	5709	$\pm$
	362	5714	$\pm$
	363	5715	-
	371	5721	$\pm$
	373	5723	-
	375	5725	$\pm$
	376	5726	-

TABLE 2. Revertibility Test (Cont.)

Cistron	Allele #	KSU Strain #	Revertibility
cysC	377	5727	-
	383	5733	-
	387	5737	±
	389	5739	++
	759	6106	-
	837	6222	-
cysD	51	5444	-
	62	5447	-
	60	5449	-
	68	5450	-
	71	5452	-
	72	5453	-
	76	5457	-
	79	5459	-
	82	5462	-
	84	5464	-
	118	5492	++ (sp)
	245	5605	+
	251	5611	-
	275	5630	±
cysE	199	5570	++
	264	5619	+
	396	5746	+
cysG	385	5735	++
cysJ	299	5652	++
ilvA	2	7149	++
	3	7150	++
	6	7153	++
	11	7158	-
	12	7159	++
	13	7160	-
	14	7161	-
	15	7162	+++ (sp)
	16	7163	-
	17	7164	+++ (sp)
	18	7165	-
	19	7166	-
	20	7167	-
	21	7168	-
	23	7170	-
	25	7172	+
	26	7173	++
	27	7174	++
	28	7175	-
	29	7176	++
	30	7177	+

TABLE 2. Revertibility Test (Cont.)

Cistron	Allele #	KSU Strain #	Revertibility
ilvA	31	7178	+
	32	7179	+
	33	7180	++
	34	7181	+++ (sp)
	35	7182	-
	36	7183	++
	39	7186	-
	40	7187	+++ (sp)
	41	7188	++
	42	7189	+
	43	7190	+
	44	7191	±
	45	7192	++ (sp)
	46	7193	+
	47	7194	++
ilvB	7	7230	+
	9	7232	++
	10	7233	-
	15	7238	-
	18	7241	-
	49	7270	+
	56	7276	-
	62	7282	++
ilvB	64	7284	+±
	65	7285	++ (sp)
	67	7287	-
	72	7292	++
	73	7293	-
	74	7294	+±
ilvC	13	7236	-
	16	7239	-
lys	6	1619	++
phe	30	8515	++
pyrE	9	2862	-
	11	2864	-
	17	2870	-
	20	2873	+++ (sp)
	22	2875	+++ (sp)
	26	2879	+++ (sp)
	50	2903	+++ (sp)
	84	2937	+ (sp)
	123	2976	-
	141	2994	-
	143	2996	-
serA	4	9001	-
	5	9002	+++ (sp)

TABLE 2. Revertibility Test (Cont.)

Cistron	Allele #	KSU Strain #	Revertibility	Cistron	Allele #	KSU Strain #	Revertibility
serA	6	9003	+++ (sp)	serA	279	9274	-
	7	9004	+++ (sp)		60	9034	-
	8	9005	-	thrB	60	9366	-
	9	9006	-	thy	53	2002	-
	10	9007	±		54	2003	-
	11	9008	-		55	2004	-
	12	9009	-		56	2005	-
	13	9010	-		57	2006	+
	27	9013	-		58	2007	+
	29	9014	-		59	2008	-
	39	9021	-		60	2009	+++ (sp)
	55	9029	+++ (sp)		61	2010	+
	61	9035	-		62	2011	-
	64	9038	-		63	2012	+
	77	9045	-		64	2013	+++ (sp)
	83	9050	-		65	2014	-
	84	9051	-		66	2015	++
	86	9053	+±		68	2017	-
	94	9061	-		69	2018	+++ (sp)
	100	9067	-		70	2019	+++ (sp)
	232	9207	-		71	2020	+++ (sp)
	274	9269	-		72	2021	+++ (sp)
	275	9270	+++ (sp)		73	2022	-

TABLE 2. Revertibility Test (Cont.)

Cistron	Allele #	KSU Strain #	Revertibility	Cistron	Allele #	KSU Strain #	Revertibility
thy	74	2023	±	metC	57	8055	+++ (sp)
	75	2024	+++ (sp)		90	8087	-
	77	2026	+++ (sp)		378	8369	-
	78	2027	+++ (sp)				
			Multiple Mutants				
pyr	209	3415	+	met	158	8153	++
tyr		"	-	trpD		"	-
argE		"	-	his		8535	+++ (sp)
purE	51	4600	++	phe	27	"	-
trpD		"	+	serA	270	9266	-
purE		4601	++	aroC		"	-
trpD		"	+	serA	272	9267	-
cysD	51	5444	++	tyr		"	-
trpA		"	+	argE		"	-

Designations:

- ; no reversion

+ or ++ ; moderate reversion

+++ ; heavy reversion

(sp) ; appearance of many spontaneous revertants.

EXPLANATION OF PLATE I

Growth curves showing synchronization after transfer of log phase culture to a fresh medium. Cells were grown to stationary phase. Portions were removed at indicated times (A: 10 to 20 min after the culture had reached stationary phase, B: 50 to 80 min). Cells from B (B) showed good synchronization, but this was not the case in (A).

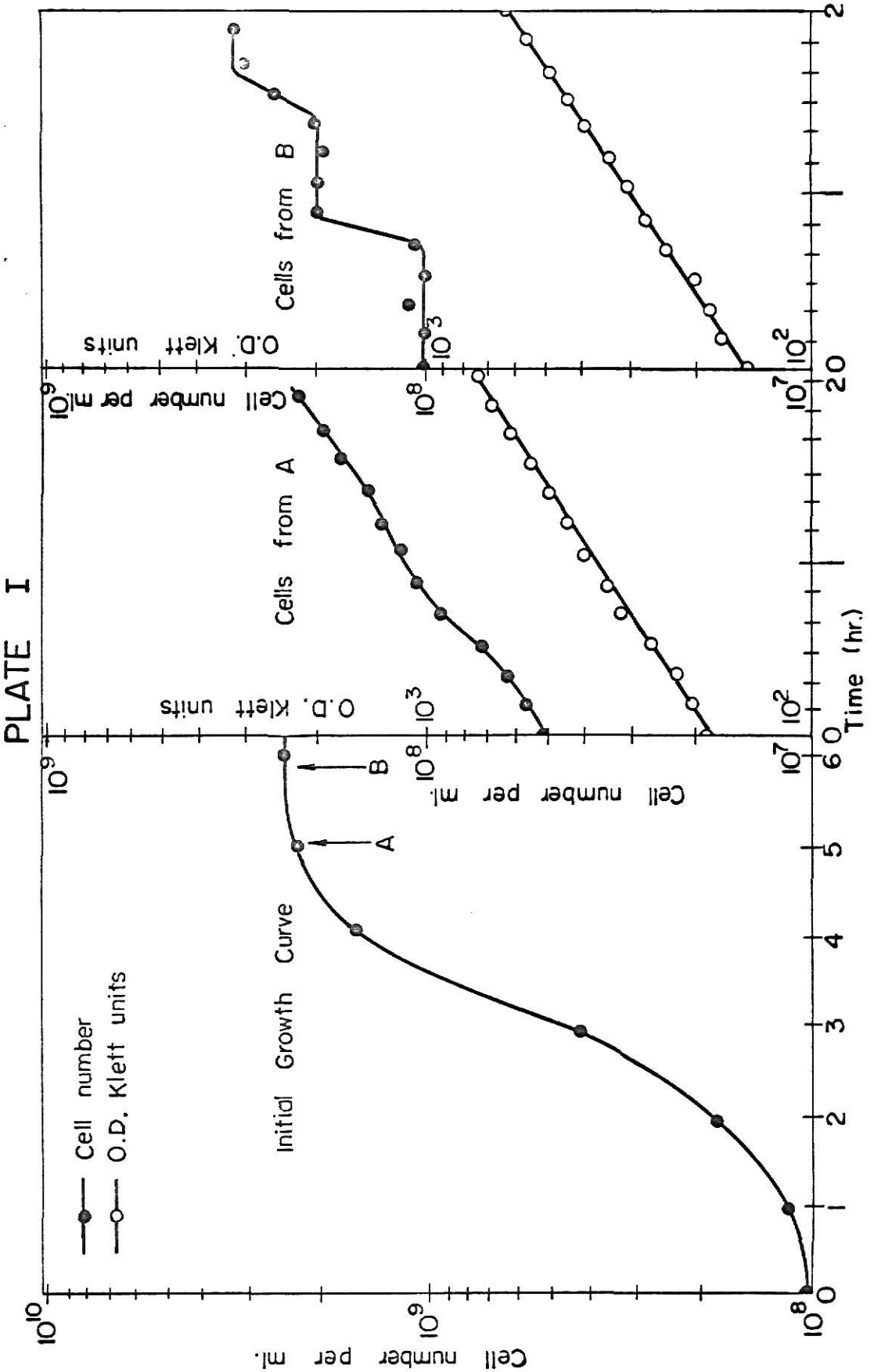

Fig. 1. Growth curves showing synchronization after transfer of log phase cells to a fresh medium.

mental steps for this determination.

Cells from the stationary phase culture were transferred into fresh medium with amino acid to start the synchronous growth. After 20 min of growth, a portion of the culture was filtered, washed, and transferred into medium without amino acid to limit the replication cycle to only one. Fig. 2a shows that synchronous growth occured in the presence of amino acid after transfer, and also that no cell division was observed in the absence of amino acid. For an early marker, cysG, the revertant peaks appeared at early stages of each replication cycle in the presence of amino acid. When amino acid was withdrawn at 20 min, no increase of cell number was observed but the number of revertants increased abruptly up to 120 min and then decreased again (Fig. 2b). For a late mutant marker, lys, in the presence of amino acid, revertants peaks appeared at late stages in each cycle, but in the absence of amino acid a peak appeared only at a late stage of the first replication cycle (Fig. 2c). These results suggest that NG incorporated into cells acts preferentially on the early genes when replication starts after plating on fresh medium.

From these results of Fig. 2a, 2b, and 2c, it may be seen that it is possible to determine the replication origin by comparing the ratios of number of revertants at log phase and at stationary phase (stationary/log) using various markers. This was tested for a number of markers and the results are shown in Table 3. As may be seen, the ratios are divided into three groups: (1) the high ratio group (argE, cysG, cysE, cysC, cysJ), (2) the low ratio group (lys, thy, and phe), and (3) the intermediate ratio group between (1) and (2) (purE, trpD, and his). Group (1) would represent the early markers, the group (2) would correspond to the late markers, and group (3) would be the middle markers. If the results are compared with the genetic linkage map shown in Fig. 3, most of the markers locate on the map as expected except cysC, cysJ, and ilvA. These

EXPLANATION OF PLATE II, III and IV

Residual mutagenic effect of NG. A portion of the stationary phase culture was transferred into fresh medium. After 20 min, a portion of the culture was filtered, washed and transferred into amino acid starved medium to limit the replication cycle to only one.

Plate II. Growth curve of transferred cells in the presence and absence of amino acid.

Plate III. CysG (an early replicating marker) cells were sequentially mutagenized with NG at 10 min intervals and the revertants were scored after two days incubation.

Plate IV. Same procedure was followed for lys (late replicating marker) cells.

 in the presence of amino acid.

 in the absence of amino acid.

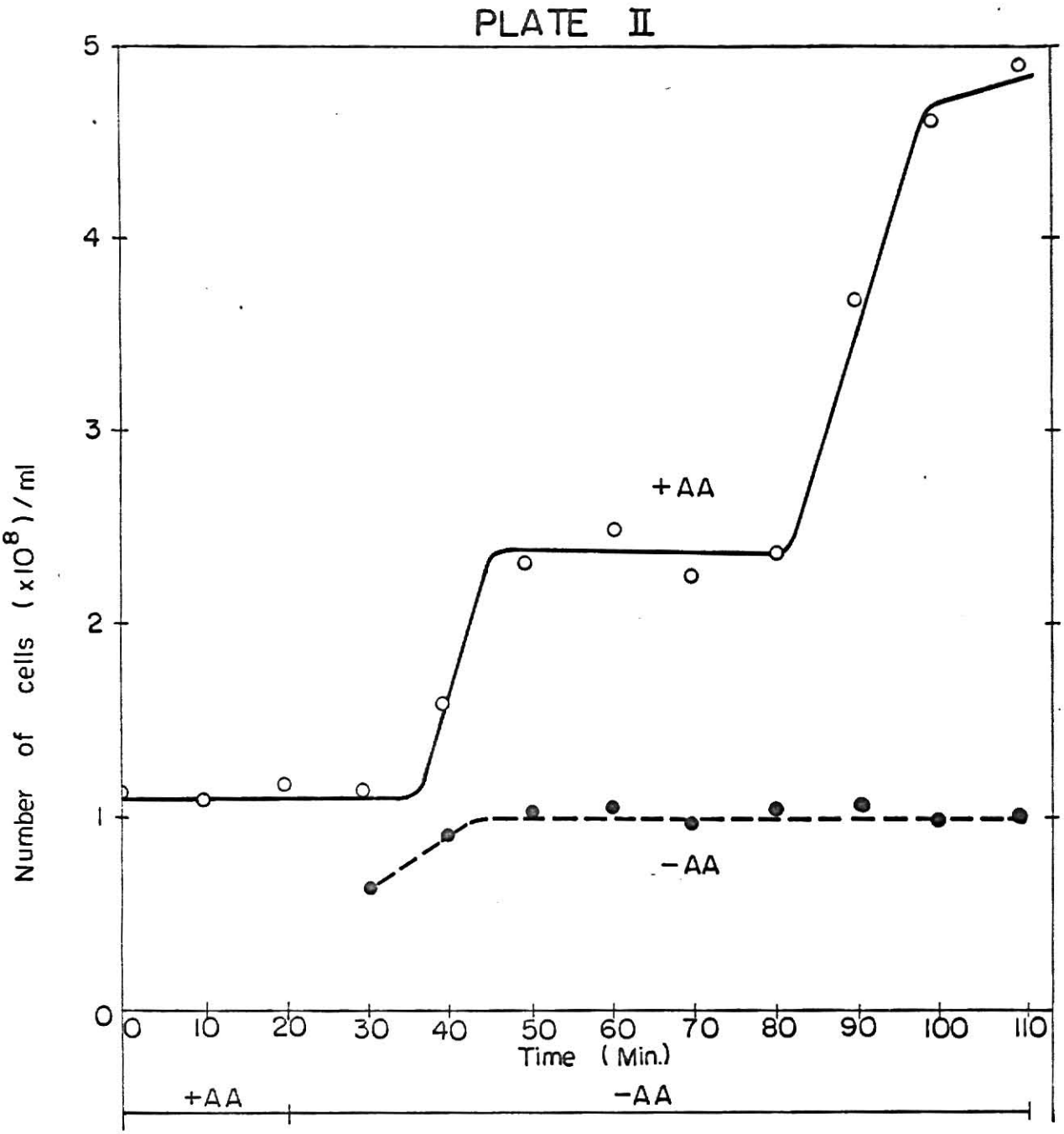

Fig. 2a. Growth curve after transferring the stationary phase culture into fresh medium with and without amino acid.

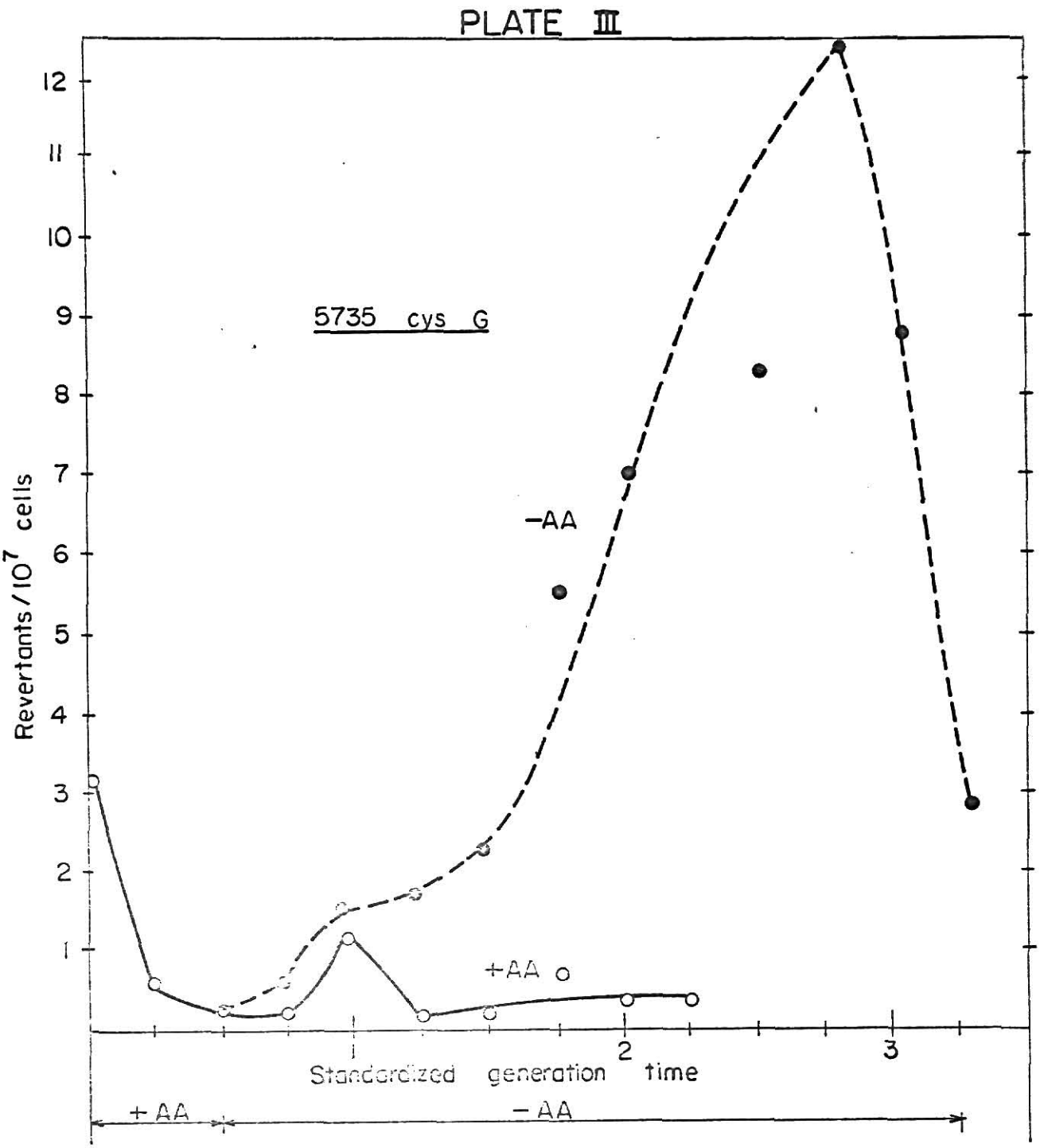

Fig. 2b. Residual effect of NG on an early replicating gene.

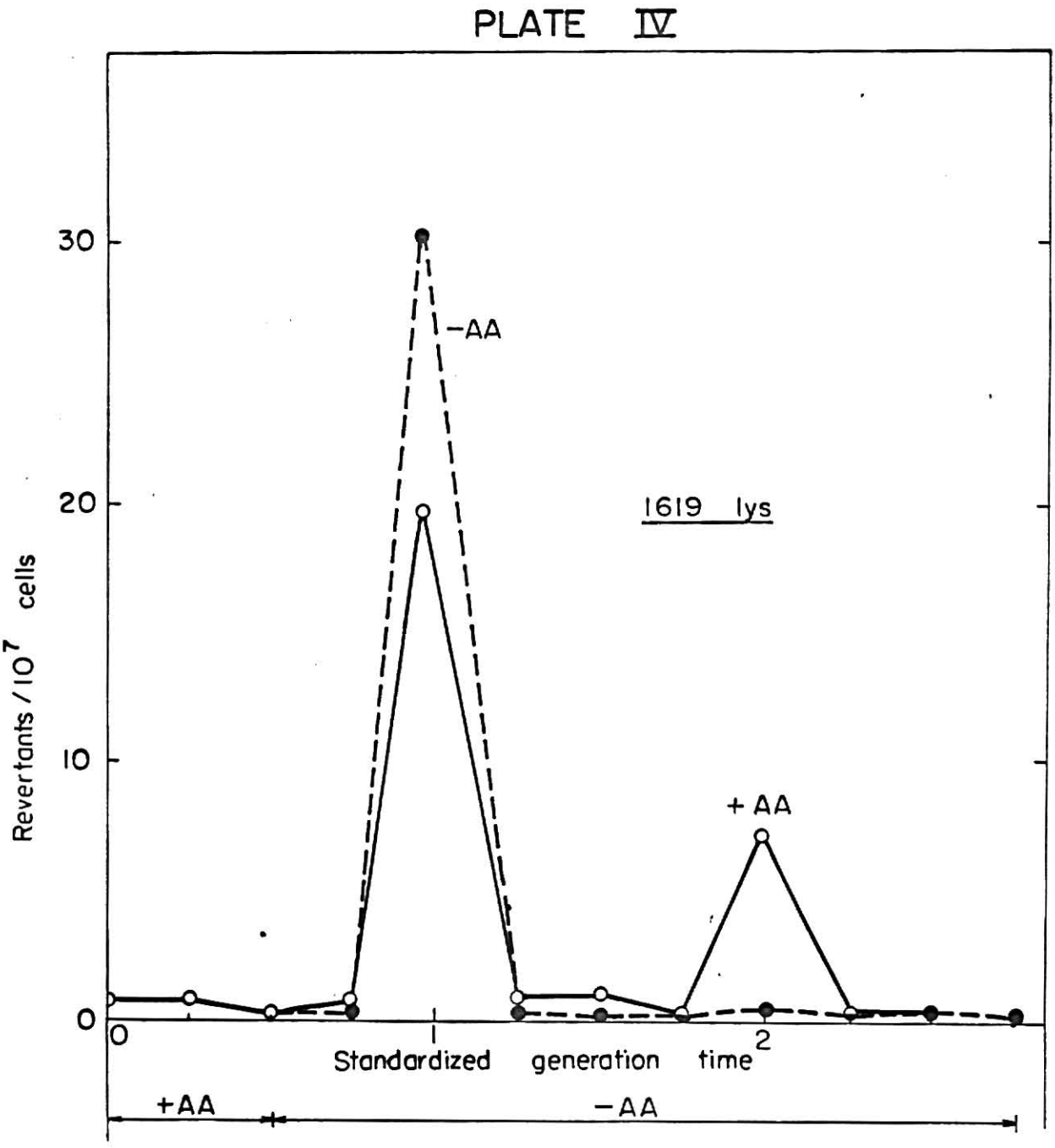

Fig.2 c. Residual effect of NG on a late replicating gene.

TABLE 3. Comparison of Number of Revertants at Stationary Phase and Log Phase

Cistron	KSU Strain #	# of revertants/10^8 cells** S-phase	Log phase	Ratios of revertants stationary/log
argE	5012	15.39	2.29	6.90
cysG	5735	41.81	1.96	21.34
cysH	5570	107.40	7.45	14.36
cysC	5604	3.71	0.77	4.82*
	5739	35.56	2.13	16.70*
cysJ	5652	144.00	6.90	20.88*
lys	1919	12.73	12.68	1.00
thy	2015	10.89	10.48	1.04
phe	8515	15.92	15.08	1.06
ilvA	7159	13.50	58.10	0.23*
	7174	27.10	43.80	0.62*
purE	4601	86.30	26.20	3.29
trpD	4601	3.70	1.20	3.08
his	6452	30.30	13.50	2.24

* reveals the contradictory results.

** Spontaneous revertants appeared in control was negligible Comparing to the NG treated sample.

EXPLANATION OF PLATE V

The outer circle indicates the linkage map of Salmonella typhimurium. The inner circle shows the replication map obtained from NG mutagenesis experiments. The replication origin was dtermined to locate the region between argE and lys and the direction of it to be colckwise as shown in figure.

PLATE V

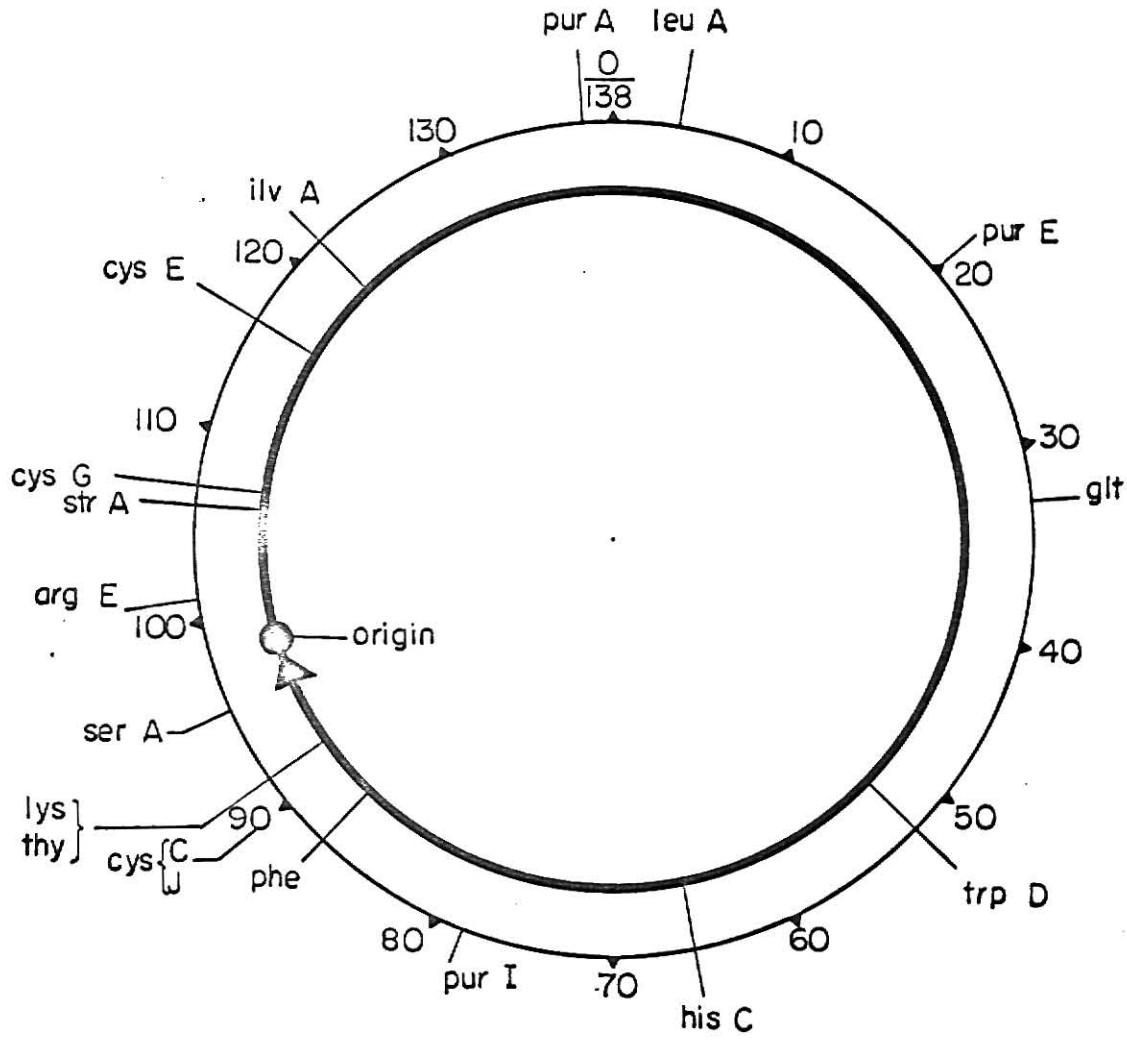

Fig. 3. Linkage and replication map of Salmonella typhimurium.

discrepancies are not understood, but some possibilities will be considered in the discussion. The direction of replication was indicated by noting the intermediate ratios of the middle markers.

iv) Sequential Mutagenesis

Based on the NG property of preferential mutagenesis at the chromosomal growing point, it is also possible to determine the replication origin and direction by mutagenizing the synchronized cells at various intervals (Cérda-Olmedo et al., 1968). The mutation frequency of a particular marker should increase greatly if NG is added at the time that the growing point is rolling through this region of the chromosome.

The results of sequential mutagenesis experiments are shown in Fig. 4. The first peak for each marker appeared at the anticipated time in a replication cycle, depending on the mutant used. The replication time is designated by measuring the interval between the first peak and the second peak. This is roughly equivalent to generation time, which is measured as a doubling of colony-forming cells. The replication time under conditions used was about 45 min and therefore this was used as a standard to compare the position of peaks that appeared in a replication cycle.

These results agree well, on the whole, with the former results in which the revertant ratio of stationary/log phase cells were used (Table 3). An exception is cysJ. CysJ seems to be an early marker in the ratio experiment but a late marker in the sequential mutagenesis experiment. The discrepancy will be discussed later. The linkage map and replication map obtained by this sequential mutagenesis experiment are in agreement, indicating the linkage map reflects the physical replication map (Fig. 3).

EXPLANATION OF PLATE VI

Sequential mutagenesis of S. typhimurium. Portions of synchronized culture were treated with NG at 10 min intervals. Revertants for nutritional requirements were scored. Replication cycle time was standardized by considering that the time between first and second peaks is the replication cycle time.

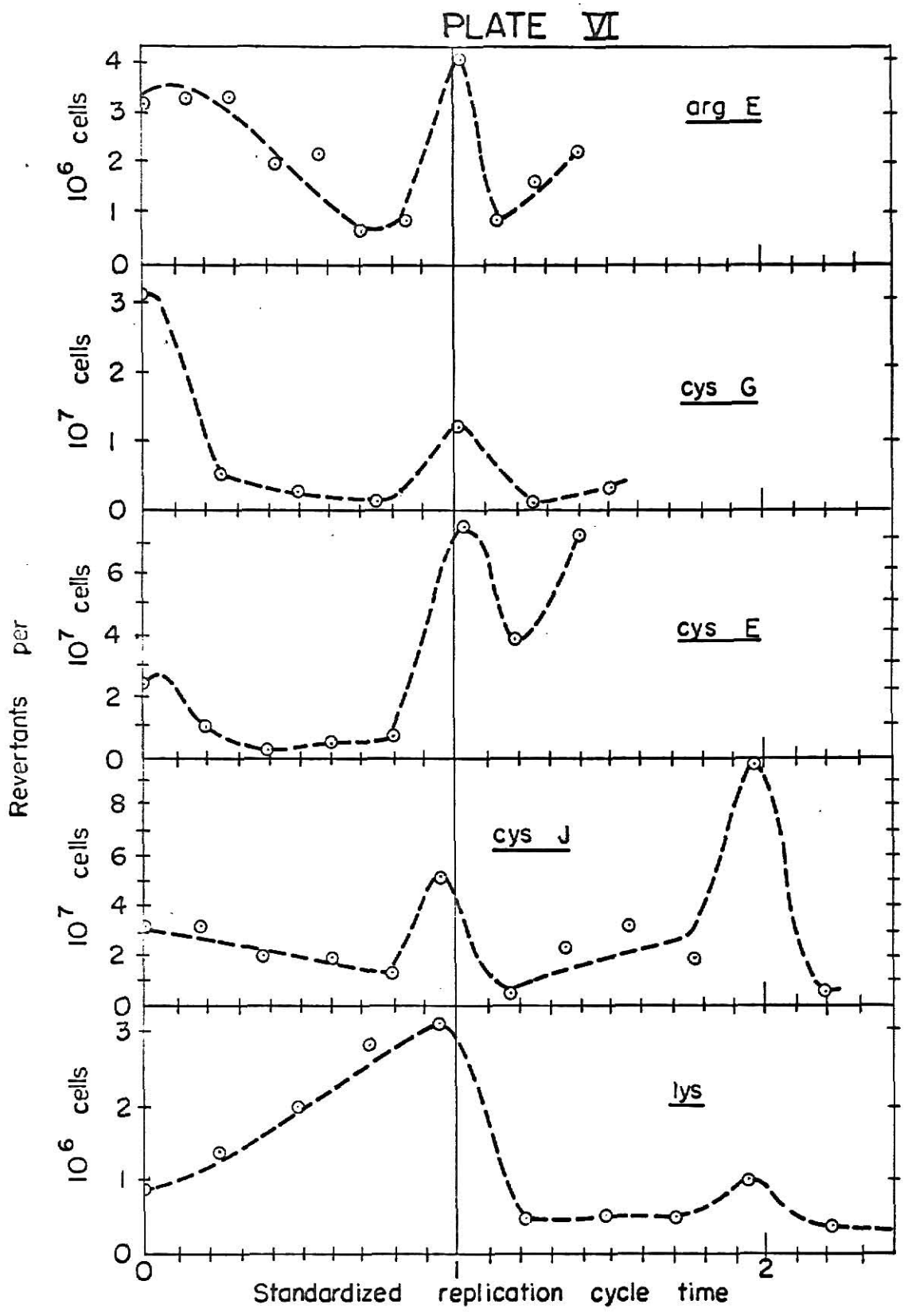

Fig. 4. Sequential mutagenesis.

DISCUSSION

i) Synchronization

Physiological synchronization is extremely useful for studies such as those described here. Using the Cutler and Evans method (1966), in which they obtained synchronous growth of E. coli and Proteus vulgaris, synchronization was observed in S. typhimurium at least two generations. There was no difference whether the stationary phase culture was directly diluted into the fresh medium or washed once and then diluted.

An indication that synchrony was satisfactory is the observation of high reversion rate of a presumably early marker, cysG, when NG is pulsed into a culture at the beginning of a synchrony cycle. As shown in Table 3, the ratio of revertants at log phase and at stationary phase (stationary/log) in cysG is 21.34. The value indicates that a high percentage of the cells in the population were synchronized, based on our assumption that NG reversion occur preferentially along a growing chromosome.

In a comparison of different methods of synchrony that was made as a preliminary to their study, it was difficult to maintain the synchronization as described by Helmstetter and Cummings (1964). Since it was very easy to obtain the physiological synchronization by the Cutler and Evans method, this procedure was adopted.

ii) NG Mutagenesis

The preferential effect of NG on the replication point of the chromosome was reported by Cérda-Olmedo et al. (1968). They compared the level of mutagenesis in cells taken from log phase culture and cells in which DNA replication cycle had been completed by amino acid starvation. Their expectation was that the former chromosomes have replication point but the latter do not, and in all

cases more revertants appeared in log phase cells than in the cells in which replication cycle was completed. With the same expectation as theirs, we mutagenized the cells at log phase and at stationary phase, but we obtained rather different results. The ratios of the number of revertants (stationary/log) change depending on the location of the markers. Therefore, we speculated that the residual NG incorporated in cells at stationary phase acts preferentially on the early markers because of synchronous growth mentioned above. This was confirmed experimentally (experiments with similar rationale has been reported by Botstein (1969)). Appropriate mutants, cys G and lys, were transferred from the stationary phase into the fresh medium and mutagenized with NG at 10 min intervals. As shown in Fig. 2b and 2c, a portion of culture was deprived of amino acid. Under this condtion the new replication cycle was not induced. In an early marker, cysG, the number of revertants increased even after the one cycle was finished but did not in lys. These facts suggested that the residual NG could effect on an early marker when the synchronous growth occurs after plating but could not retain its effect on a late marker.

The difference in ratios of revertants (stationary/log) will give the region of replication origin. In other words, the early genes have high ratios and the late genes the low ratios. Table 3 shows that argE is an early gene and lys is a late marker in the comparison experiments. From this, it seems that the replication starts somewhere between these two genes in a clockwise direction, since the intermediate ratios were obtained from the middle markers.

We consider that this method is more effective to determine the replication origin than the sequential mutagenesis to be mentioned next. However, we have unexplainable results, namely the contradictly ratios of cysC, cysJ,and ilvA. This could be due to the large NG pool of these strains, the heterogeneity of the replication pattern even in the derivatives of the same strain, that muta-

tion occurs in supressor genes which might be early genes, or the mutation in episome existing near these genes. The third possibility is unlikely because of the consistent results obtained by repeated experiments both in the same different strains with mutations in the same cistron.

During the time that my study was under way, Botstein and Jones (1969) reported the difference in the location of mutagenized gene with NG between at log phase and at right after the stationary phase in E. coli. My results are roughly consistent with theirs.

Cérda-Olmedo et al. (1968) reported that the sequential mutagenesis is a relatively convenient method to determine the replication origin and direction, especially if the good synchronization is obtained. By this method they found the periodical replication of DNA, the fixed origin between arg and tyr on the chromosome map and the fixed clockwise direction of replication, although map positions of markers they used were not definitive. They also found that in all strains tested (E. coli 15T⁻, K12 Hfr and K12 F⁻) the fixed origin was determined on the map. Using this technique we tried to determine the replication origin and direction. Figure 4 shows the results. ArgE, cysG and cysE are all early markers, while lys and cysJ are late genes. The results are different for cysJ between the comparison experiment (stationary/log) and the sequential mutagenesis. This discrepancy is not also understood. While the results are still not conclusive, they favor a clockwise unidirectional model of replication starting from the region between argE and lys (Fig. 4).

Nishioka (1969) considered several models, including that ilvA is close to the origin and the replication proceeds in bidirectional way. He synchronized cells with amino acid starvation and also thymine starvation. The cultures were grown in the presence of BU at early, middle and late stage of a replication cycle respectively. From the transducing efficiency of bacteriophage P22

containing BU, he determined the fixed origin and the fixed bidirectional replication. There are two alternative possibilities, (1) the replication really proceeds in a bidirectional manner (2) the results were artifacts caused by the treatments, amino acid starvation, thymine starvation, and/or BU incorporation. A similar phenomenon was reported by Caro and Berg (1969). But they concluded it as artifacts caused by amino acid starvation or BU treatment. The results obtained by the physiological synchronization should represent the more actual situation in bacteria.

Another factor that makes one hesitate to draw firm conclusion is that NG effects are not completely understood. The wide range of mutagenesis of NG makes it difficult to specify the properties of it. However, our experiments also indicate that NG preferentially acts on the replication point of chromosome. But it is not clear whether NG causes methylation or deamination of a single stranded region of replication point of DNA or attacks the replication machinery such as DNA polymerase or ligase. The activity of E. coli DNA polymerase purchased was not effected by NG (Eisenstark and Consigli, 1969, pers. comm.). But it is not known that NG acts on DNA polymerase in vivo.

SUMMARY

Log phase cultures and stationary phase cultures were treated with nitrosoguanidine (NG) respectively. The ratios of revertants at stationary phase and log phase (stationary/log) were examined, using various markers. When the early markers were selected, high ratios were obtained, and low ratios were obtained from the late markers. The intermediate ratios corresponded to the selected markers located between early and late genes. NG incorporated into cells at stationary phase should act more effectively on early genes than at log phase, because a new round of DNA replication occurs after transfer of the stationary phase culture into fresh medium. In contrast, at log phase, the replication point of the chromosome is scattered over the entire chromosome, making a marker gradient of frequency between early and late genes. The results indicated that *argE* is the early marker and *lys* is the late marker. Sequential mutagenesis was performed in parallel. The portions of the synchronized culture of an appropriate strain were treated with NG at various times and the revertants were selected. Both of these results showed good agreement on the whole: the replication of the chromosome is initiated between 8 and 9 o'clock on the genetic map and proceeds clockwise.

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DISTINCTION BETWEEN EARLY AND LATE GENES REPLICATED IN SALMONELLA TYPHIMURIUM, AS DETERMINED BY NITROSOGUANIDINE MUTAGENESIS

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ABSTRACT

The purpose of this investigation was to identify the region of the genetic map of Salmonella typhimurium at which chromosome replication originates, and the direction in which it proceeds. A number of studies have been devoted to this problem with Escherichia coli and Bacillus subtilis.

Basically, two experimental systems were used. One involved a comparison of marker revertibility between stationary phase cells and log phase cells. The other method involved revertibility of markers upon sequential mutagenesis in synchronized cultures, as described by Cérda-Olmedo et al. (1968). In both experiments, nitrosoguanidine (NG) was used as the mutagen. The rationale in both types of experiments is that NG mutations occurs preferentially at the growing point of the replicating chromosome.

In first experiment, stationary phase cells were treated with NG and placed in a growth medium. There is a greater probability of mutaion for genes located near the origin of chromosome replication, because syncronous growth occurs after transterring the stationary phase culture into a fresh medium. In contrast, in log phase cells, each gene has an equal opportunity for exposure to the mutagen. Thus, ratios of the number of revertants at stationary and log phase (stationary/log) were examined, scoring for auxotrophic revertants scattered aroud the chromosome. The ratio was high for markers near 9 o'clock (e.g., cysG) on the S. typhimurium genetic map, and low for markers near 8 o'clock (e.g., lys). Markers that were distant from these (12 to 6 o'clock) yielded intermediate ratios.

In the second method, synchronized cells were pulsed with NG at various times after initiation of replication cycles. Appropriate auxotrophic markers were scored for reversions. The results confirmed that there is preferred

reversion of cysG soon after initiation of cycle, but lys does not reach its peak of NG reversion until later in the replication cycle.

The results obtained from two different experiments support the model that, in S. typhimurium, chromosome replication is initiated 8 to 9 o'clock on the map and proceeds clockwise.