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Regulation of Chromosome Replication and Segregation in Bacteria

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INTRODUCTION

A knowledge of the mechanisms which regulate cell growth and division has long been accepted as a prerequisite to understanding the development and differentiation of biological systems.

Of all the synthetic events which occur within a cell during its division cycle, none achieves the importance of chromosome replication. Without this, the information for the regulated construction of cell material cannot be transferred to future generations. It is not surprising, therefore, that in a broad spectrum of biological systems, ranging from bacteria to mammals, interference with the replication of genetic material invariably results in the cessation of cell division.

For several years our laboratory has been interested in the relationship between deoxyribonucleic acid (DNA) synthesis and cell division. At first this interest took the form of experiments on synchronized cultures of bacteria in which the inhibition of DNA synthesis could be related to cell division, or vice versa. The results of these studies may be summarized as follows. (i) Inhibition of DNA synthesis blocked cell division, whereas inhibition of cell division did not necessarily inhibit DNA synthesis (25). (ii) The timing of DNA synthesis in the cell division cycle was regulated in some manner which did not solely depend upon the act of chromosome replication (15). (iii) Production of the precursors required for DNA biosynthesis was not fundamental to the regulation of the timing, or rate, of chromosome replication (16). (iv) Regulation of the pattern of DNA synthesis (both its timing and rate) could be drastically altered by changing the conditions for cell growth (13, 22, 32).

These results led us to study the regulation of sequential chromosome replication and its relation to cell growth and division. The present article reviews our experiments of the past 3 years and, in particular, those of the past 12 months. Some of these, which have not yet been published, will be discussed in greater detail than others. (In the figures and tables the former will be noted as "*unpublished data*" the latter as "*from published data*")

From these experiments we have drawn the following conclusions. (i) The pattern of chromosome replication changes as the rate of cell division is reduced; i.e., the rate of replication becomes slower, replication occupies a smaller

fraction of the division cycle, and the number of chromosomes per cell is reduced. (ii) Chromosome segregation at intermediate rates of cell division follows a pattern in which each of the two chromosomes in the cell is derived from a different parent chromosome. Moreover, this is done in such a manner as to distinguish between the two strands of each parent chromosome which served as templates during replication. (iii) A heritable region of the chromosome exists, *the origin* from which each cycle of sequential chromosome replication is initiated. (iv) Thymine starvation prematurely induces sequential replication from the chromosome "origin." However, this premature replication is restricted to one of the two "origins" available on each replicating chromosome. (v) Two processes, each requiring the participation of amino acids, are involved in initiating a cycle of chromosome replication. One of these is inhibited by chloramphenicol and 5-fluorouracil; the other is not. Below, I will present a new model of chromosome replication based on these conclusions.

My discussion of the experiments will be divided into two sections: those relating the pattern of chromosome replication and segregation to cell division, and those relevant to the mechanisms regulating chromosome replication.

PATTERN OF CHROMOSOME REPLICATION AND SEGREGATION

Chromosome Content at Different Growth Rates

Previous studies by Schaechter et al. (32, 33) demonstrated that the DNA content of individual cells of *Salmonella typhimurium* decreased as the growth rate decreased, and that DNA synthesis continued throughout the division cycle when cells grew with a generation time of 40 min or less.

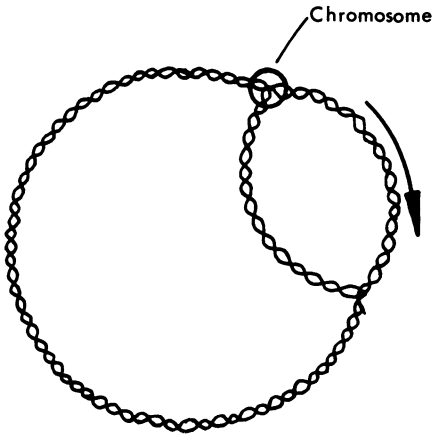

FIG. 1. *The circular chromosome of Escherichia coli (5). The direction of replication is indicated by the arrow. This structure is diagrammatic and is not intended to imply any mechanism of replication. (See, however, Fig. 24.)*

TABLE 1. *DNA content of Escherichia coli 15T⁻ grown in different media [data of C. Lark, reproduced from: Biochim. Biophys. Acta (12)]*

Energy source	Doubling time	DNA content*
	min	
Glucose	40	0.138
Succinate	70	0.103
Aspartate	120	0.070
Acetate (15T⁻-AR) †	120	0.073
Proline	180	0.051
Acetate	270	0.055
Replicating *E. coli* chromosome (5)		0.065
Nonreplicating *E. coli* chromosome (5)		0.045

* Expressed as micrograms per 10^7 bacteria.

† A strain of 15T⁻ selected for its ability to grow on acetate medium with a 2-hr generation time was designated as 15T⁻-AR (fast). The values given are for this strain, grown on acetate medium.

A similar study was made by C. Lark (12), correlating the chromosome content per cell and the chromosome replication pattern with the division rate. For this she used *Escherichia coli* 15T⁻, a quadruple auxotroph which requires thymine, tryptophan, methionine, and arginine. This organism has been used in all of the experiments to be described.*

By preparing radioautographs of intact *E. coli* chromosomes, Cairns (5) demonstrated their circular structure (Fig. 1). From his pictures it could be estimated that an intact nonreplicating chromosome had a mass corresponding to 0.45 $\times 10^{-14}$ g of DNA. This corresponds to an average value of 0.65 $\times 10^{-14}$ g of DNA for chromosomes in the process of replication (assuming exponential growth of the culture and a constant rate of chromosome replication throughout the cell division cycle).

Table 1 presents data of C. Lark (12) on the relation of cellular DNA content to the rate at which cells are growing. They suggest that, at rapid growth rates, each cell may possess two or more chromosomes, whereas at slower rates they contain only one.

* The materials and methodology involved in these experiments were described in a number of articles. Specific aspects are: growth of 15T⁻ and transfer to different media (23, 31), density gradient centrifugation (23, 31), use of radioisotopes (23, 31), radioautography (12, 19, 20), and properties and use of inhibitors (14).

This is supported by radioautographic data for cells which contained DNA labeled with H^3-thymine, and which were allowed to divide in nonradioactive medium. As cells divide they distribute the four conserved pieces of DNA (8, 12, 19, 20, 37) which make up each replicating chromosome, over the progeny population; after several generations of growth, each of the progeny cells contains either one radioactive piece of DNA or none. Such experiments (12, 19) indicate that cells growing in glucose or succinate medium contain between seven and eight conserved pieces of DNA, corresponding to four double-stranded units of DNA or two replicating chromosomes. On the other hand, a similar analysis (12) has shown that there are about one-third as many conserved units of DNA per acetate-grown cell. This result is consistent with the data in Table 1, and indicates that, in the cells of an acetate-grown culture, the chromosomes do not replicate during the entire division cycle.

It should be noted that in autoradiography each cell is identified cytologically and counted as a cellular unit.

Chromosome Replication and the Cell Division Rate

It is obvious from the data in Table 1 that the average rate of chromosome replication must decrease as the growth rate decreases, since the amount of material per cell decreases and is duplicated over a longer average time interval. Nevertheless, it is possible that a rapid rate of replication of each individual chromosome could be maintained if each chromosome is replicated during only a portion of the cell division cycle, as observed in cells of higher organisms (17). This has been tested by allowing cells to incorporate radioactive thymine into their chromosome for a fraction (about $\frac{1}{10}$) of a generation period and then preparing autoradiographs to determine how many cells are labeled, i.e., are in the process of replicating their chromosome. The results in table 2 indicate that at longer generation times, cells do not replicate their chromosomes throughout their entire division cycle. It is also clear that, as the generation period increases, the duration of replication of an individual chromosome also requires a longer time.

The results obtained from cells growing slowly appear to be straightforward, since their content of DNA indicates that they possess only one chromosome. However, both glucose-grown and succinate-grown cultures appear to contain two

TABLE 2. *Chromosome replication in different media (from published data) (12, 19)**

Medium	Generation period	Unlabeled cells	Chromosome replication time	No. of chromosomes made
	min	%	*min*	
Glucose	40	1	40	2
Succinate	70–75	6	70	2
Aspartate	120	10	110	1
Proline	180	42	110	1
Acetate	270	41	160	1

* The percentage of the cell population not synthesizing DNA was measured by autoradiography of pulse-labeled cells. From the generation period, the time spent in chromosome replication was calculated. Autoradiographs were analyzed when the average number of grains was estimated to be between 20 and 30 per cell.

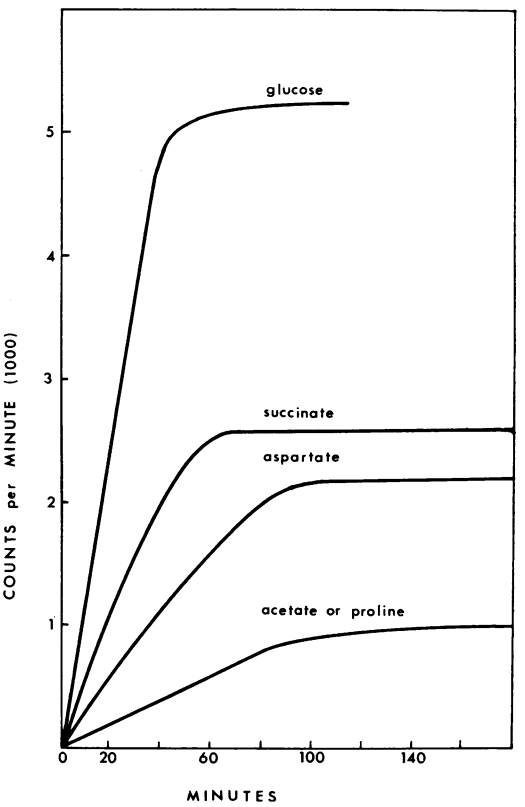

FIG. 2. *The synthesis of DNA in the absence of required amino acids (from published data) (5, 20). Incorporation of H^3-thymine per 10^7 cells was measured (ordinate) as a function of the time of incubation in the absence of amino acids (abscissa). The results with cultures growing in five different media are represented. Radioactivity of 5,000 counts per min per 10^7 cells represented the synthesis of an amount of DNA equal to 40% of the DNA present at 0 min.*

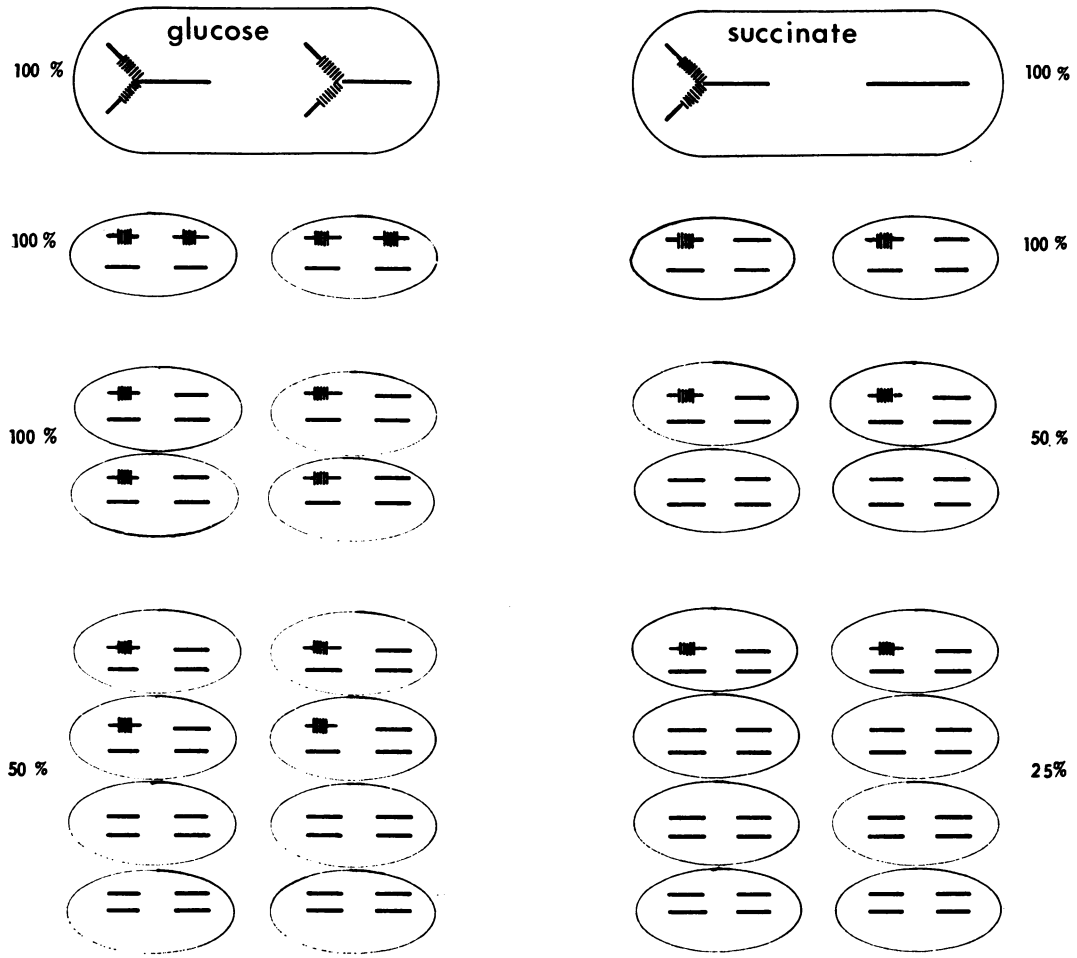

FIG. 3. *Model for the cellular distribution of pulse-labeled radioactive DNA during subsequent generations of growth. The distribution for cells growing in glucose or in succinate medium is shown. The predicted frequency of labeled cells at each generation is indicated at the margin. In this model a chromosome is represented by a single line.*

chromosomes per cell, and an interpretation of their replication pattern is therefore more complicated.

Further insight into their replication pattern was obtained when we measured the amount of DNA which could be synthesized in the absence of amino acids by the cultures listed in Tables 1 and 2. As we shall see in a subsequent section, in the absence of required amino acids replication of a chromosome is completed, but a new cycle of replication cannot be initiated. Therefore, the amount of DNA which is made under these conditions is a measure of the number of chromosomes which are in the process of replication. Figure 2 presents the incorporation of radioactive thymine in the absence of amino acids by cultures growing in the indicated media.* It can be seen that such cultures fall into three groups: glucose; succinate and aspartate; acetate and proline. These data are most easily reconciled with those in Tables 1 and 2 if: (i) in glucose cultures, the two chromosomes in each cell are replicated simultaneously throughout the division cycle; (ii) in succinate cultures, one of the two chromosomes in each cell is being replicated at any instant, but the replication of both occupies the entire generation period; (iii) in aspartate cultures, one chromosome is being replicated throughout

* For simplicity, experimental points are not reproduced on the curves of Fig. 2 and certain others taken from already published articles.

the division cycle; (iv) in proline or acetate cultures, only half of the cells are in the process of replicating their single chromosome at any instant.

The data in Tables 1 and 2 and in Fig. 2 substantiate this description of chromosome replication in aspartate, proline, and acetate cultures. Moreover, the DNA content of proline and acetate cultures is lower than expected for a single replicating chromosome, yet greater than expected for a nonreplicating chromosome. This suggests that those cells in which chromosomes are not replicating contain only one (not two) chromosomes. This is supported by radioautographic studies (12) in which it was found that half of the cells from acetate and proline cultures grown for many generations on H^3-thymine contain only two conserved pieces of DNA (or one finished chromosome), whereas the other half contain four (or one replicating chromosome). These facts lead to the conclusion that in such slow-growing cultures chromosome replication occurs at the end, rather than the beginning, of the cell division cycle. The significance of this will be discussed later.

The hypothesis that in glucose cultures two chromosomes within each cell replicate simultaneously, whereas cells from succinate cultures replicate one of their two chromosomes at a time, is borne out by autoradiography (19, 20). Cells from both types of culture were allowed to incorporate a small amount of H^3-thymine, and were then placed in nonradioactive medium to divide. After one, two, three, and four generations, samples were taken and the number of labeled cells was estimated. Figure 3 shows the distribution of radioactive cells according to the hypothesis.

TABLE 3. *Distribution of pulse-labeled radioactive DNA in daughter cells during successive generations of growth (From published data) (20)**

Generations after labeling	Percentage of cells with no label	
	Glucose	Succinate
0	<1	6
1	<1	13
2	5	28
3	22	60
4	45	78

* After a pulse of H^3-thymine, exponential cultures were allowed to grow in nonradioactive medium; samples were taken immediately, and when the population had doubled, quadrupled, etc. Population growth was estimated within 3% by continual monitoring with a Coulter counter. Samples were analyzed by autoradiography and scored when the average number of grains per cell was between 20 and 30.

TABLE 4. *Distribution of pulse-labeled radioactive DNA into daughter cells during successive generations of growth (from published data) (19)**

Generations after labeling	Percentage excess of lightly labeled cells lying outside of distribution	
	Glucose	Succinate
0	0	5
1	2	4.3
2	7	49
3	49	72
4	67	87

* The experiment was carried out in the same manner as in Table 3, except that the analysis of radioautographs was performed after a shorter exposure, when the grain count had reached between two and three per cell in each sample. Samples were analyzed by use of the Poisson distribution: $\log [P(r)r!] = r \log n - (n/2.3)$, where $P(r)$ is the probability of finding r grains per cell and n is the average number of grains per cell. A graph such as that in Fig. 5, in which values of $\log P(r)r!$ are plotted as a function of r, will yield a straight line if the distribution fits a Poisson distribution. Cells receiving no DNA or only a fragment of DNA will deviate from this distribution, exaggerating the values of $P(r)r!$ observed at low values of r. By extrapolating the linear portion of the graph, this excess of lightly labeled or unlabeled cells can be measured, furnishing an estimate of the percentage of cells lying outside of the distribution. The slope of the linear portion of the graph will yield the average number of grains per cell for cells presumed to lie within the distribution. In the experiments presented here (19), the average number of grains per radioactive cell decreased by factors of two at each of the first two divisions of the glucose culture, but the average grain count per radioactive succinate-grown cell was halved only at the first division. The average grain count per radioactive cell remained constant in subsequent division of the glucose or succinate cultures.

Tables 3 and 4 give the results of two types of experiments demonstrating that the distribution shown in Fig. 3 is obtained. I shall discuss these experiments in detail since they and others like them form the basis of several conclusions in this and the next two sections. In the experiment shown in Table 3, cells were labeled with a short pulse of H^3-thymine and then allowed to grow in nonradioactive medium. The increase in cell number was carefully measured in a Coulter counter, and, when the numbers of cells had

doubled, a sample was taken for radioautography, i.e., after exactly one generation of growth. Further samples were taken after two, three, and four generations of growth. These were examined by autoradiography in such a way that each radioactive cell would produce an average of 20 to 30 grains in the emulsion. The number of cells with no grains was then counted. It may be seen, that this number rises suddenly between two and three generations of growth of the glucose culture. On the other hand, a similar rise (correcting for the number of unlabeled cells initially present) occurs between the first and second generation in the succinate culture. This is the behavior predicted from the model in Fig. 3. However, in neither case does the number of unlabeled cells increase to 50%. This has been attributed to chromosome fragmentation (such as sister strand exchanges) and the subsequent distribution of these fragments into cells which would otherwise not receive label. Forro has presented experimental evidence for such fragmentation (7, 8).

The experiment in Table 4 escapes the complication of fragmentation by including all of the cells in the distribution. This is done by exposing the autoradiographs for a short period of time until an average of two to three grains per cell is obtained. The number of grains associated with each cell is then measured and the distribution compared with a Poisson distribution (expected if all of the cells possess an equal amount of radioactive DNA). This comparison is made in a graph of log $[P(r)r!]$ versus r, as in Fig. 4 where $P(r)$ is the probability of obtaining r grains per cell (19, 37). A simple Poisson distribution is indicated by a straight line. Cells with no radioactive DNA or receiving a small fragment of radioactive DNA will lie outside of this distribution, causing an upward deviation of log $[P(r)r!]$ from this straight line. The number of such cells can be measured. Table 4 (19) presents this type of analysis of an experiment similar in other respects to that described in Table 3.

The proportion of cells receiving no radioactive DNA or a fragment of radioactive DNA agrees well with the frequency predicted from the model in Fig. 3.

An additional item of information derived from this experiment (19) was the interesting finding that the amount of radioactivity incorporated per minute per conserved unit of DNA was almost the same for glucose and for succinate cultures, indicating that in both of these cultures the rate at which an individual chromosome replicates is about the same. Further data relating to this were given by Lark and Bird (19).

We have concluded that: at fast growth rates (glucose medium) each cell contains two chromosomes, both of which are replicating simultaneously throughout the division cycle; at somewhat slower growth rates (succinate medium) the cell again contains two chromosomes, one of which is being replicated at any instant; at still slower growth rates each cell contains a single chromosome which may be replicated throughout the division cycle (aspartate medium); and at very slow growth rates (proline or acetate medium) this single chromosome is replicated during the last half of the division cycle. It is important to note that as the growth rate decreases the duration of the replication of a single chromosome increases (from 35 to 40 min in glucose or succinate medium to 135 min in acetate medium).

TABLE 5. *Distribution into daughter cells of radioactive DNA, pulse-labeled at intervals after a medium change (unpublished data)**

Time of labeling after medium change	Generations after labeling	Glucose into succinate, per cent of cells with no grains	Succinate into glucose, per cent of cells with no grains
min			
0	2	7	23
	3	23	43
10	2	11	20
	3	28	48
20	2	8	22
	3	28	44
30	2	17	15
	3	33	33
40	2	28	12
	3	43	30
50	2		4
	3		28
60	2	27	
	3	49	
70	2		6
	3		20

* An exponential-phase glucose-grown culture was transferred to succinate medium, and was labeled by treatment for 5 min with H^3-thymine (100 μc/ml; 2 μg/ml) at intervals. Each labeled sample was grown in nonradioactive medium and sampled after a four- or eightfold increase in cell number. A similar experiment was carried out after a shift from succinate to glucose medium. Each sample was analyzed by autoradiography after exposure sufficient to yield an average of 20 to 30 grains per cell.

TABLE 6. *Distribution into daughter cells of radioactive DNA, pulse-labeled at intervals after a medium change (unpublished data)**

Time of labeling after medium shift	Generations after labeling	Per cent outside distribution	
		Glucose to succinate	Succinate to glucose
min			
0	1	—	1
	2	1	46
	3	51	—
25	1	—	3
	2	1	46
	3	42	—
40	1	1	—
	2	43	22
	3	—	60

* This experiment was performed in the same way as that shown in Table 5, except that samples for autoradiography were exposed for shorter intervals (yielding an average number of two to three grains per cell). Samples of the distribution obtained are shown in Fig. 4. The data are the result of an analysis of the type used in Table 4.

Changing the Pattern of Chromosome Replication by Shifts Between Glucose and Succinate Media

Table 3 shows that in a culture with a glucose type of chromosome replication pattern the percentage of unlabeled cells increased from 5 to 22% between the second and third generations. In a culture with a succinate chromosome replication pattern, the percentage of unlabeled cells increased from 28 to 60% between the second and third generations.

When a culture growing in glucose medium is shifted to succinate medium, or vice versa, the chromosome replication pattern must change. This, however, does not occur immediately. Table 5 presents results of an experiment, similar to that in Table 3, in which cells growing in glucose were shifted to succinate medium and cells growing in succinate medium were shifted to glucose medium. Different portions of each culture were pulse-labeled (5 min) with radioactive thymine at different intervals after the medium shift. They were then allowed to grow for two or three generations in nonradioactive medium, and long-exposure autoradiographs were prepared.

It is clear from Table 5 that, after a shift from glucose to succinate medium, chromosome replication continues to follow the glucose pattern for at least 20 min, after which it changes to that observed for growth in succinate. The change is complete by about 40 min. Similarly, after a culture is transferred from succinate to glucose medium, the succinate chromosome replication pattern persists for at least 20 min, after which it gradually changes to the glucose pattern. This change is completed by about 50 min after the transfer into glucose.

These conclusions are strengthened by another experiment (Table 6). Cells were transferred from glucose to succinate medium or from succinate to glucose medium, and were pulse-labeled with H^3-thymine immediately or after 25 or 40 min. They were then grown in nonradioactive medium and, after one, two, or three generations of growth, samples were taken for autoradiography. The number of grains per cell was counted and the distribution calculated. Samples of these distributions for the glucose to succinate transfer are shown in Fig. 4. The results, which may be interpreted by comparison with those in Table 4, are shown in Table 6. These confirm the results in Table 5 and demonstrate that the chromosome replication pattern changed between 25 and 40 min after the shift to succinate medium, and between 25 and sometime after 40 min after the shift to glucose medium.

The autoradiographic data in Tables 5 and 6 are substantiated by measurements of the amount of DNA which can be made in the absence of amino acids. As I already have mentioned (and will be discussed in detail subsequently), the amount of DNA which can be made in the absence of amino acids is a reflection of the number of chromosomes in the process of replication. Table 7 gives the amount of DNA made in the absence of amino acids at intervals following a shift from glucose to succinate medium or vice versa. This decreases to reach a constant amount 40 to 50 min after the shift from glucose to succinate medium. Conversely, it increases after a shift from succinate to glucose medium, and almost reaches the amount characteristic for a glucose culture by 60 min after the shift.

The data in Tables 5, 6, and 7 demonstrate that the chromosome replication pattern is not altered immediately after a change in medium. Instead, they suggest that after a change in growth medium cells tend to complete a replication cycle before changing their chromosome replication pattern. This is supported by an experiment in which cells growing in glucose medium containing D_2O-$N^{15}H_4Cl$ were shifted to a succinate medium containing H_2O-$N^{14}H_4Cl$. This density change was also made when shifting cells from succinate to glucose. By measuring the heavy, hybrid, and light DNA, an estimate was obtained of the amount of nonreplicated, once-replicated, and twice-replicated DNA. Figure 5 shows the syn-

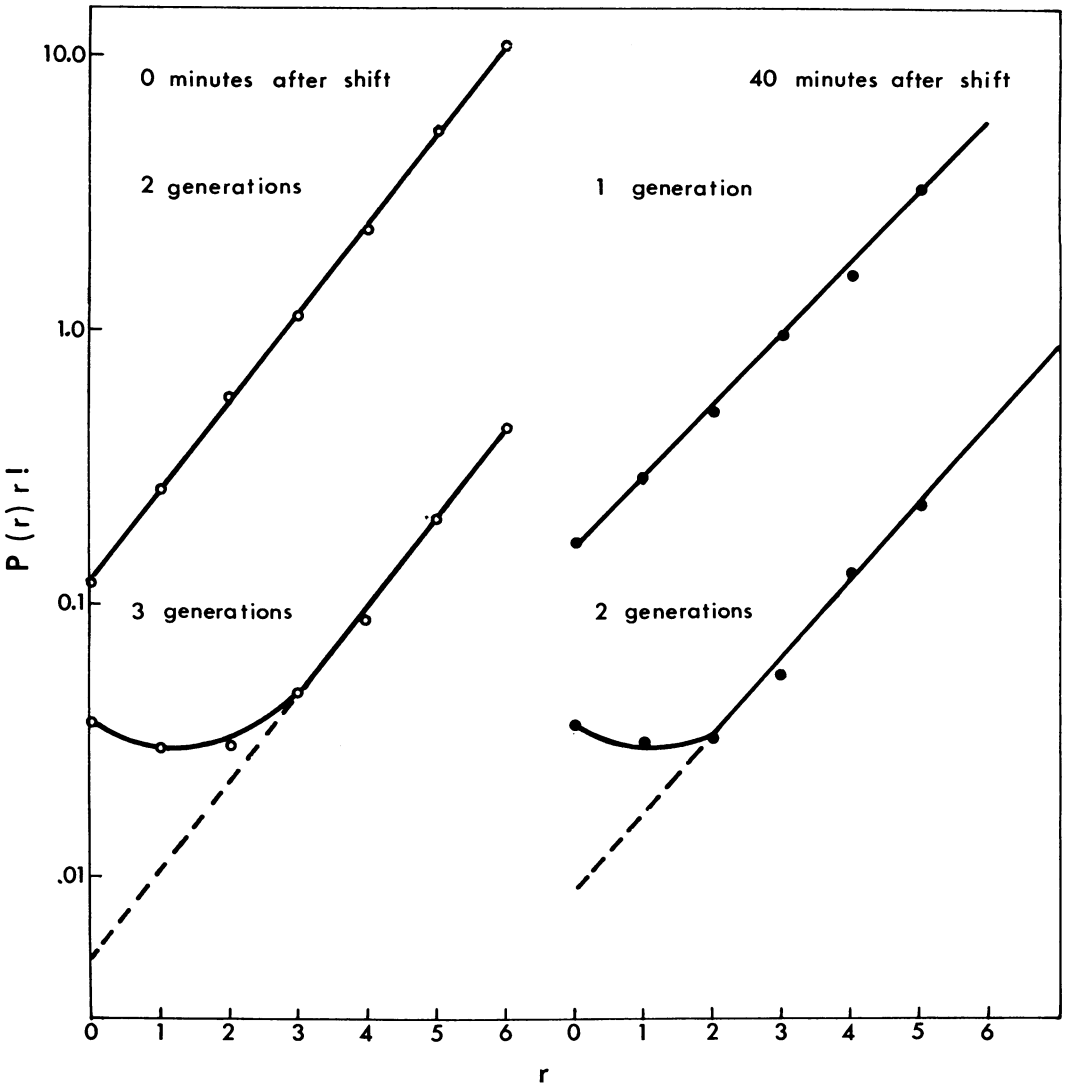

FIG. 4. *Distribution of the number of grains per cell in a culture which had been pulse-labeled with H^3-thymine immediately after, or 40 min after, transfer from glucose to succinate medium (unpublished data). Samples were removed for autoradiography after one, two, or three generations of growth in nonradioactive medium. The form of the Poisson distribution used is: log [P(r)r!] = log n − (n/2.3). A more detailed description of this experiment is presented in Table 6. The method of graphing and analyzing the results is given in Table 4.*

thesis of these types of DNA expected according to a model in which chromosome replication is completed before a change in replication pattern can occur.

The results of the experiment are given in Table 8. They compare well with what would be expected on the basis of the model in Fig. 5. For example, for the shift from succinate to glucose one can predict that 11% of the original DNA will have replicated twice when between 75 and 80% has been replicated at least once. Similarly, after a shift into succinate 16% of the DNA will have replicated twice when between 60 and 65% of the original DNA will have replicated at least once.

The results in Tables 5–8 are consistent with the model in Fig. 5.

Chromosome Segregation

The data presented in Tables 4 and 6, and in Fig. 4, allow us to draw certain conclusions about the segregation of the two chromosomes in cells growing in succinate medium.

TABLE 7. *Synthesis of DNA in the absence of amino acids following a medium shift (unpublished data)**

Time after shift from glucose to succinate	Increase in DNA in absence of amino acids	Time after shift from succinate to glucose	Increase in DNA in absence of amino acids
min	%	*min*	%
0	42	0	19
10	39	10	22
20	31	20	24
30	23	30	25
40	18	40	27
50	16	50	29
60	17	60	34

* An exponential glucose culture of $15T^-$ (2×10^8 cells per milliliter) was transferred to succinate, and portions were deprived of amino acids in the presence of H^3-thymine (10 μc/ml; 2 μg/ml). Incorporation of radioactivity was measured. In all cases this incorporation followed the pattern shown in Fig. 2. The final amount of radioactivity incorporated was expressed as the percentage of the amount incorporated by a glucose culture (2×10^8 cells per milliliter) during one generation of exponential growth. Another experiment was performed in which a culture was shifted from succinate into glucose medium.

In such cells, a pulse of radioactivity labels the progeny only of that chromosome which is in the process of replication (Table 4). These might be segregated from unlabeled chromosomes at the next division, or they might not, as shown in Fig. 6. If, in all cells, the progeny of the one chromosome are necessarily separated from the progeny of the other (Fig. 6A), then half of the cells in the first generation will be unlabeled and half will retain the radioactive content of the parent cell. On the other hand, if both labeled progeny of one chromosome and an unlabeled progeny of the other are distributed to each daughter cell (Fig. 6B), then all of the first-generation cells will be labeled and the radioactive content per cell will be half that of the parent. Table 4 demonstrates that the second model (Fig. 6B) is correct. Moreover, not only were essentially all of the cells radioactive after the first division, but the average grain count per radioactive cell was found to drop by about one-half, from 4.14 to 1.79 (19). These data demonstrate that in succinate-grown cells segregation of the progeny of one chromosome from those of the other does not occur and that the distribution of these progeny is not random.

The segregation of chromosomes in glucose-grown cells cannot be determined by this type of experiment, since a pulse of radioactivity will label all of the progeny of both parent chromosomes.

However, when a chromosome is transferred from glucose to succinate medium, both chromosomes are replicating simultaneously. Therefore, a pulse of radioactive thymine, administered immediately after the shift, will label all of the new strands of both chromosomes (Fig. 7). After one generation of growth in succinate medium, each daughter cell will contain two radioactive chromosomes, one in the process of replication and the other waiting to be replicated. When both have replicated, each cell will contain two labeled and two unlabeled chromosomes, a labeled and unlabeled one arising from each parent chromosome. These progeny will be distributed into the second-generation daughter cells according to the segregation pattern observed for succinate cells; i.e., each daughter cell will receive one of the progeny from each of the two chromosomes present in the parent cell. [This follows from the fact that this segregation pattern is observed after one generation of postlabeling growth in cells which were pulse-labeled 40 min after the shift down (Fig. 4B and Table 6). This corresponds to a total growth of between one and two doublings after the shift down.]

We are therefore in a position to determine whether, in this second generation, chromosomes are chosen at random from among the progeny of each chromosome, since the progeny will contain one labeled and one unlabeled chromosome depending upon whether it carries a template made two generations previously or a template made during the previous generation (during radioactive labeling). If one member of the progeny of each chromosome is selected at random, we expect that 25% of the cells will receive no radioactive chromosomes, 25% will receive two, and 50% will receive one (Fig. 7, model A). If they are chosen according to a definite selection rule, whereby each daughter cell receives one chromosome which carries a template made during the previous generation and one which is built upon a template made two generations previously, then all of the cells will be equally radioactive because they all contain one radioactive chromosome (Fig. 7, model B). The results in Fig. 4 (left side) clearly demonstrate that this is the case. Moreover, after another generation of growth (third), the radioactive units are distributed into half of the daughter cells, and these labeled cells are just as radioactive as their parent (second generation) cells, indicating that the latter contained the minimal number of radioactive units possible, i.e., one conserved-unit.

Thus, from the experiments in Fig. 4 and Table 6 we can conclude that the distribution of chromo-

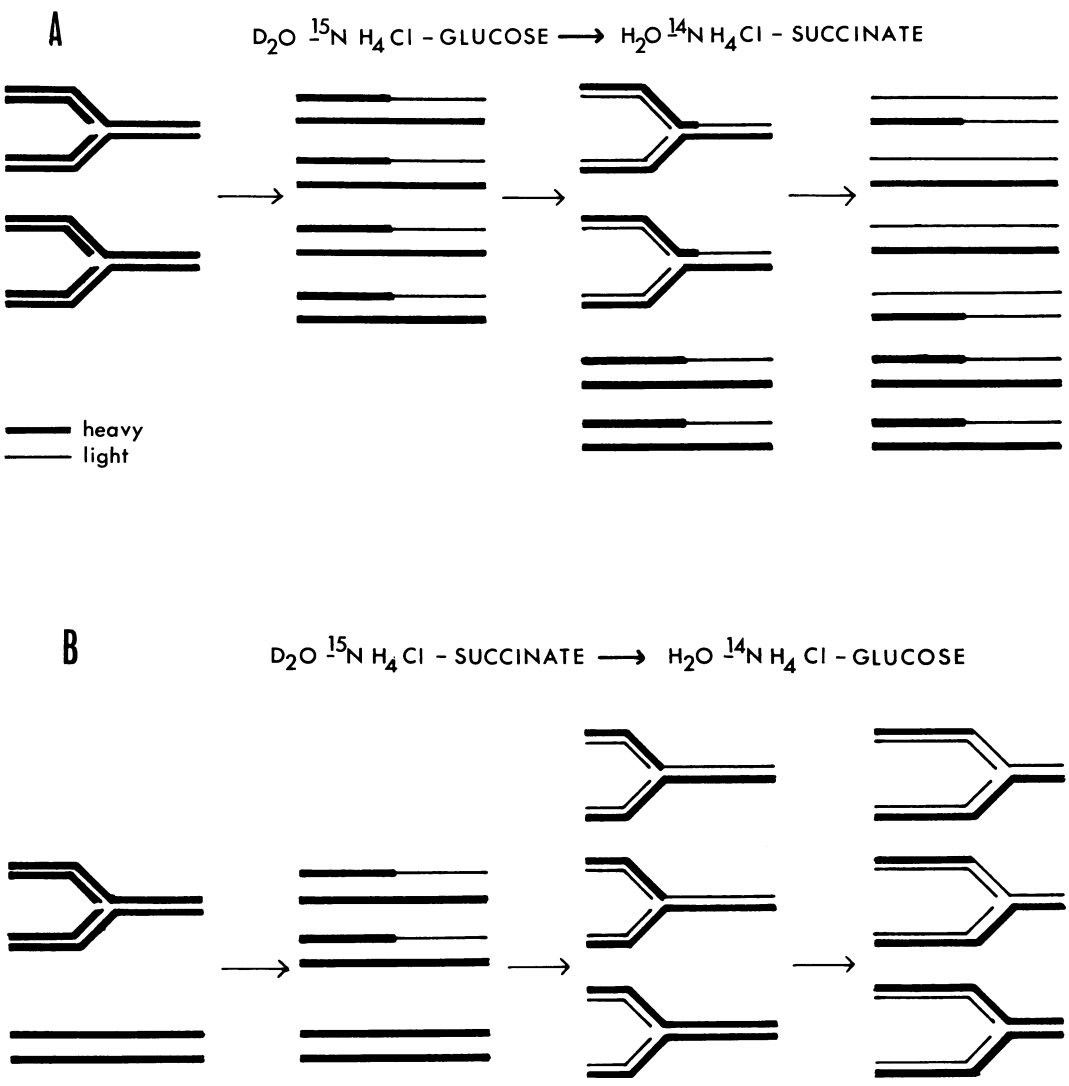

FIG. 5. *Diagrammatic representation of the chromosome replication expected after a shift from heavy succinate to light glucose medium or from heavy glucose to light succinate medium. The assumption is made that replication in progress will be completed before a change of replication pattern will occur. In Fig. 5–9, a chromosome is shown as two lines, each representing a conserved unit of DNA.*

somes among daughter cells proceeds according to selection rules which not only distribute one progeny from each chromosome into each daughter cell but actually specify which individual chromosome of the two progeny from each parent will go into a given cell.

EXPERIMENTS ON THE MECHANISM REGULATING CHROMOSOME REPLICATION

Definition of the Chromosome Origin

Effect of amino acid starvation. For some years (29) it has been known that if bacteria are starved of required amino acids a limited amount of DNA synthesis can occur (Fig. 2). Maaløe and Hanawalt (24) hypothesized that this resulted from the completion of the cycle of chromosome replication on which cells were embarked. They assumed that such cells could not begin another cycle of chromosome replication in the absence of protein synthesis (Fig. 8). They based their hypothesis on the calculation that the amount of DNA made in the absence of amino acids corresponded almost exactly to the amount expected if chromosomes, replicating sequentially

TABLE 8. *Replication of DNA after a medium shift (unpublished data)**

Shift	Time after shift	Heavy DNA	Hybrid DNA	Light DNA	Per cent of original DNA replicated at least once	Per cent of original DNA doubly replicated
	min	%	%	%		
Glucose to succinate	190	40	57	3	40	2
	200	35	60	5	46	4
	210	29	64	7	52	6
	230	20	63	17	62	16
	250	9	67	24	80	28
Succinate to glucose	60	57	43	—	28	0
	90	10	79	11	80	11
	120	1	57	42	97	73
	150	—	48	52	—	108

* A glucose culture grown in medium containing 96 atom% D_2O and 95 atom % $N^{15}H_4Cl$ was transferred to normal succinate medium. Samples were taken at intervals, lysed, and analyzed in an ultracentrifuge for the density profile of the DNA in CsCl. Another experiment, where a succinate culture growing in D_2O-$N^{15}H_4Cl$ medium was transferred into normal glucose medium, was also carried out. The percentage of replicated DNA was calculated as: ½ hybrid/(heavy + ½ hybrid). The percentage of doubly replicated original DNA was calculated as: ½ light/(heavy + ½ hybrid).

and randomly at a constant rate, were allowed to complete replication. The existence of sequential replication had been implied by the experiments of Meselson and Stahl (26) and was subsequently confirmed (5, 23, 27, 38). Maaløe and Hanawalt (24) demonstrated that, in the absence of amino acids, cells taken from a randomly dividing culture ceased to synthesize DNA at different times, a finding which also was compatible with their hypothesis.

Our initial experiments (23, 31) were directed toward testing the correctness of the Maaløe and Hanawalt hypothesis. We reasoned that, if amino acid starvation imposed a block in replication at some point on the chromosome, and if the location of this point was both fixed and heritable, it should be possible to arrive at this same point at any time by depriving the cells of amino acids. To identify this point, at two different times, we used first an isotopic and then a density label.

After a period of amino acid starvation, a culture was allowed to incorporate H^3-thymine for a short interval. Presumably, this should have labeled a portion of the chromosome near its beginning. The cells were then grown for several generations in nonradioactive medium and again starved of amino acids, after which amino acids were restored in a medium in which 5-bromouracil was substituted for thymine. The association of radioactivity with DNA of hybrid density was measured. The total increase in hybrid DNA was also measured. The latter may be taken as a measure (assuming sequential replication) of the average length of chromosome which has replicated, the relation being: fraction of DNA replicated = per cent hybrid DNA/(200 − per cent hybrid DNA).

A summary of the protocol of this experiment is shown in Fig. 9 and in Table 9, in which the events assumed to occur within the cell are outlined at each stage in the experiment. It may be seen that, if the hypothesis is correct, the density

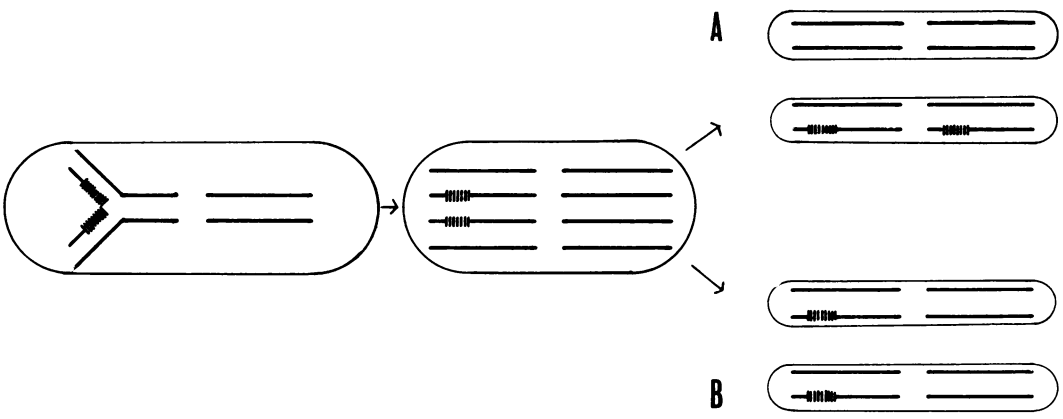

FIG. 6. *Two alternative models for the distribution of radioactive chromosomes into daughter cells (see also reference 19) for cells from a succinate culture pulse-labeled with H^3-thymine and then grown for one generation in nonradioactive medium. Radioactive segments of the chromosome are indicated by the "hash marks" in this figure and in Fig. 7, 8, and 9.*

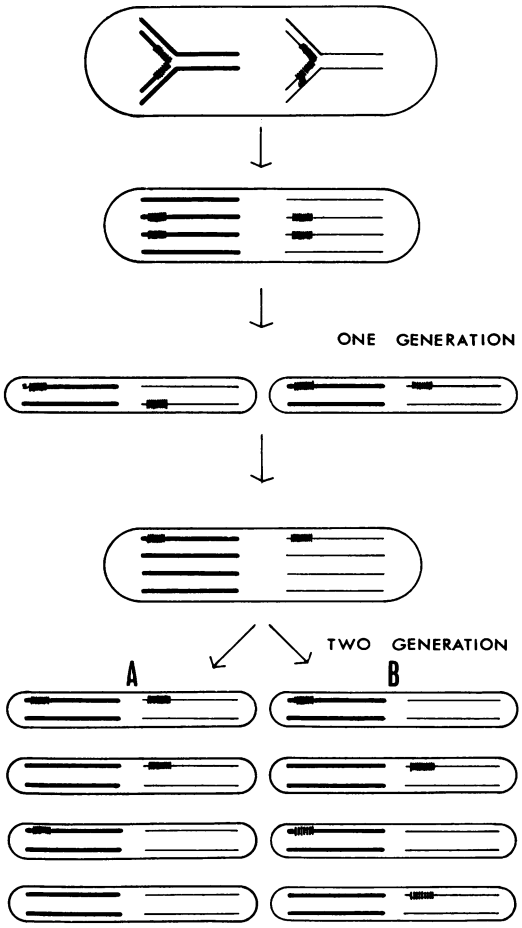

FIG. 7. *Two models for the distribution of radioactive chromosomes into daughter cells. A glucose culture was shifted into succinate medium and pulse-labeled with H^3-thymine. Two possible distributions are shown after two generations of growth in nonradioactive succinate medium have elapsed. The two parental chromosomes are distinguished by different thickness of lines.*

label should be initially incorporated into the same region of the chromosome into which the isotopic label was incorporated. This would mean, that the first DNA to become hybrid should be radioactive DNA. It can be seen in Fig. 10 (curve A) that this is what happens.

The initial incorporation of density label into the radioactive portion of the chromosome is a result of the preceding periods of amino acid starvation. Thus, if the second period of amino acid starvation is omitted, the density label is incorporated into different positions on the chromosomes of individual cells. Such a result is indicated by curve B in Fig. 10.

The chromosome replication observed following amino acid starvation is not an isolated event, but rather the commencement of sequential replication. Thus, if a net synthesis of 10% of the DNA content of the cell is allowed to take place after amino acid starvation but before allowing incorporation of a pulse of radioactive thymine, the subsequent replication of the radioactive material is delayed when amino acid starvation is repeated and cells are then transferred to medium containing bromouracil (Fig. 10, curve C). These experiments demonstrate that, following amino acid starvation, chromosome replication proceeds from a particular region of the chromosome, and that this replication is sequential.

The recognition of this region of the chromosome is a heritable property, since many generations of growth can elapse between the initial incorporation of radioactive thymine and the second treatment of amino acid starvation (followed by the incorporation of the density label). Indeed, no difference has been observed between results from experiments in which two, four, or six generations of growth are allowed to separate the two starvation treatments (14). Maaløe and Hanawalt's hypothesis was based on the assumption that chromosome replication proceeded in the absence of amino acids to reach completion and that protein synthesis was necessary to initiate a new replication cycle. This is confirmed if, in the experiment outlined in Table 9, thymine is omitted during the second period of amino acid starvation. Under these conditions, DNA synthesis cannot take place during amino acid starvation and chromosome replication should not proceed. The result of such an experiment is shown in Table 10. It can be seen that replication of the chromosome is not initiated in the region into which radioactive thymine is incorporated, if amino acid starvation takes place under conditions in which chromosome replication cannot be completed.

Effect of thymine starvation and of treatment with phenethyl alcohol. The region of the *E. coli* chromosome which is synthesized after amino acid starvation appears to have a fundamental significance. Two other completely different treatments, which inhibit chromosome replication, also are followed by replication initiated in this region. Thus, Pritchard and I (31) found that we could substitute a period of thymine starvation, which completely inhibits DNA synthesis (2, 14, 31) for the second period of amino acid starvation in the experiment described in Table 9. When thymine starvation was ended by the addition of 5-bromouracil, a replication pattern

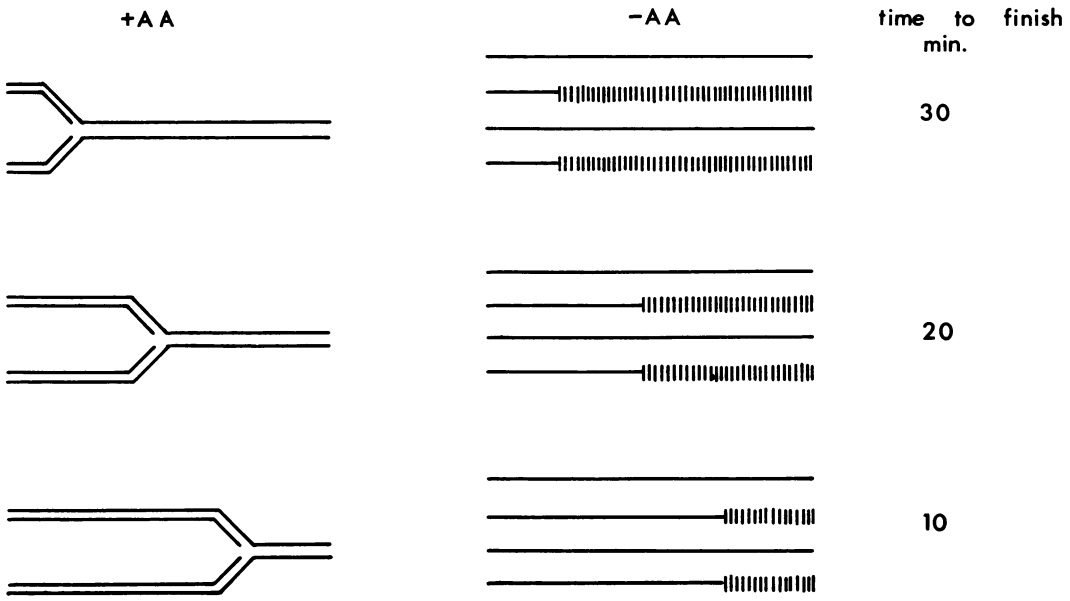

FIG. 8. *Diagram of the hypothesis of Maaløe and Hanawalt (24). Three chromosomes from a random culture are shown in different stages of replication when deprived of amino acids. It can be seen that each completes its chromosome in a different period of time. If this is done in medium containing H^3-thymine, each cell contains a different amount of radioactivity (23).*

TABLE 9. *Summary protocol of a "double-label" experiment*

Protocol step	Treatment	Presumed effect
1	− Amino acids, 80 min	All cells finish chromosomes
2	+ Amino acids + H^3-thymine	Label beginning of chromosome
3	Growth*	Randomize cycles
4	− Amino acids, 80 min	All cells finish chromosomes
5	+ Amino acids + bromouracil	Density label incorporated at chromosome beginning?

* Grown for two, four, or six generations.

was observed (Fig. 10, curve D; control, Table 10) which was similar to that observed after amino acid starvation. Thus, we were able to show that after thymine starvation chromosome replication was initiated in the region of the chromosome where it begins after amino acid starvation. The effects of thymine starvation will be discussed in greater detail in a later section.

Konetzka and his collaborators (3, 36) found that phenethyl alcohol, when added to a bacterial culture in proper concentrations, would preferentially inhibit DNA synthesis as opposed to ribonucleic acid or protein synthesis. From the pattern and amounts of DNA synthesis which they observed, they concluded that this agent acted by blocking chromosome replication at some point in the replication cycle common to all cells in the culture.

We (21) investigated whether this point is the same as that reached during amino acid starvation, by testing whether chromosome replication after phenethyl alcohol treatment recommences at the same region of the chromosome at which it is resumed after amino acid treatment. To do this, we substituted treatment with phenethyl alcohol for the first period of amino acid starvation in the protocol in Table 9. The subsequent period of amino acid starvation led to incorporation of density label into the same region of the chromosome as that into which radioactive label was incorporated after phenethyl alcohol treatment (Fig. 11).

It follows, therefore, that all three treatments—amino acid starvation, thymine starvation, or exposure to phenethyl alcohol—result in chromosome replication which proceeds from the same region of the chromosome. It seems reasonable to identify this region of the chromosome with its beginning.

Data similar to those in Fig. 10 and 12 are obtained when the experiment in Table 9 is carried out with cultures growing in succinate

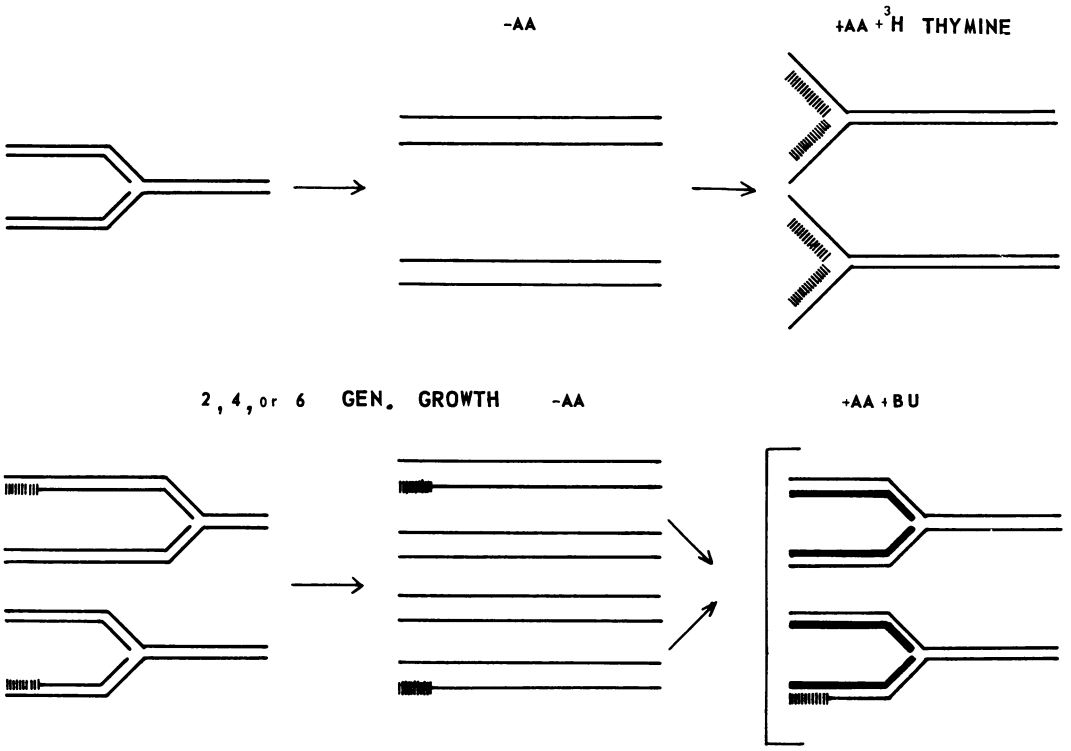

FIG. 9. *Diagrammatic representation of what should happen at each stage in the experiment described in Table 9. The heavy line represents heavy DNA containing 5-bromouracil (BU).*

medium (20). However, whereas replication begins at the chromosome origin, preferential double labeling is much less extensive than in cultures growing in glucose medium. There are two reasons for this: (i) only one of the two chromosomes in the cell will replicate immediately after amino acid starvation, and this chromosome may not bear the radioactive label (in which case the radioactive DNA will be replicated later); (ii) when cells resume chromosome replication after amino acid starvation, they do not do so simultaneously. This heterogeneity in recommencing replication is much greater in succinate cultures than in glucose cultures. In both glucose and succinate cultures, this heterogeneity is responsible for the observed reduction in preferential double labeling below that predicted. The basis for this heterogeneity also will be discussed later.

Sequential Replication

Growth in glucose medium. The sequential replication of DNA was indicated by the experiments of Meselson and Stahl (26), who measured the synthesis of DNA after a shift from a heavy (N^{15}) to a light (N^{14}) density growth medium. At the end of one generation, all of the heavy DNA had been converted to a hybrid form, whereafter light DNA began to be observed. This indicated that in every cell of the culture all of the DNA had replicated at least once, before any portion had replicated a second time. This sequential pattern of chromosome replication was confirmed in a number of experiments (4, 5, 23, 27, 38), the most striking being those of Cairns (5) in which the structure of the replicating chromosome, its sequence of replication, and its rate of replication were demonstrated by autoradiography.

We have seen (Fig. 10, curves A and C) that replication from the chromosome origin proceeds sequentially. Another type of experiment confirms this sequential chromosome replication. If cells from glucose cultures in the exponential phase of growth are exposed to a short (0.1 generation time) pulse of H^{3}-thymine and then immediately placed in medium in which 5-bromouracil replaces thymine, the cells proceed to synthesize hybrid DNA. However, the replication of radioactive DNA does not occur until almost all of the chromosome has replicated. This is seen in Fig. 12 (curve A), which shows that the con-

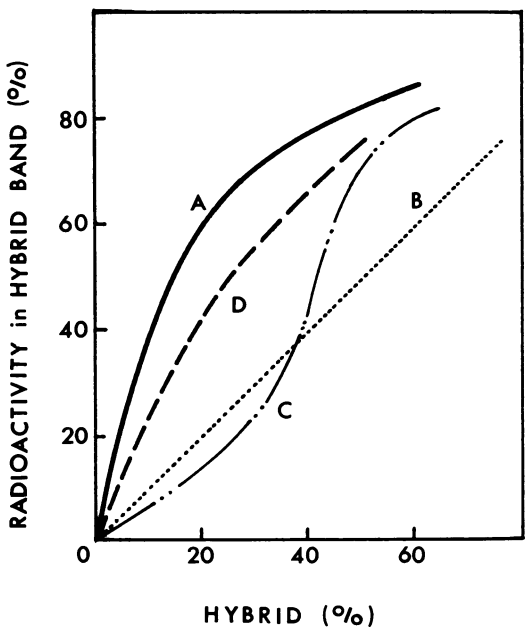

FIG. 10. *Results from experiments of the type outlined in Fig. 9 and Table 9 (from published data). (A) An experiment (31) conducted exactly as described in Table 9. (B) An experiment (14, 31) as in Table 9, in which step 4 was omitted. (C) An experiment (23) in which labeling with H³-thymine (step 2) occurred after replication of 10% of the chromosome had occurred in nonradioactive medium; i.e., the label was inserted into a region of the chromosome about 10% of its length away from the origin. (D) An experiment (31) as in Table 9, in which 40 min of thymine starvation was substituted for step 4 (the second period of amino acid starvation).*

version of the radioactive DNA into a material of hybrid density occurs very late in the overall synthesis of hybrid DNA. Curve B is the theoretical curve which would be expected if all of the cells replicated their chromosomes at the same linear rate. About 50% of the prelabeled material is replicated somewhat prematurely. In view of the evidence for sequential replication, this premature replication is most easily explained by assuming that some cells have completed their replication cycles before others (heterogeneity of the cell population with respect to chromosome replication rate). Whether this is a result of exposure to 5-bromouracil or is an inherent characteristic of the population has not been determined.

If this same experiment is repeated, but a period of amino acid starvation is interposed between the pulse of H³-thymine and growth in 5-bromouracil, replication of the radioactive portions of the chromosome follows a different pattern (Fig. 13, curve A). The H³-thymine is incorporated into different positions on the chromosomes of different cells, since the cells of the exponential culture are at different stages of their chromosome replication cycle. Starvation for amino acids in the presence of nonradioactive thymine results in the completion of chromosome replication in all of the cells. When amino acids are restored in the presence of 5-bromouracil, replication commences from the origin of every chromosome. In some cells the radioactive portion of the chromosome is near the origin, whereas in others it is further away. Thus, in some cells radioactive DNA will be converted to material of hybrid density immediately, whereas in others this will occur after a considerable period of chromosome replication. The theoretical replication pattern expected on the assumption that sequential chromosome replication commences simultaneously from the origin of all of the cells and subsequently proceeds at a constant rate is also shown in Fig. 13 (curve B). The departure of the experimental curve from this expected value is understood and will be discussed later.

These experiments emphasize once again the difference between the random location of chromosomal sites at which H³-thymine or 5-bromouracil may be taken up by exponentially growing cells, and the fixed positions into which they are

TABLE 10. *Effect of amino acid deprivation on the initiation of DNA replication by thymine starvation (from published data) (31)**

Time in bromouracil	Control (− thymine + amino acids)		Experimental (− thymine − amino acids)	
	Hybrid DNA	H³ in hybrid DNA	Hybrid DNA	H³ in hybrid DNA
min	%	%	%	%
5	8	19	7	5
12	21	44	9	11
19	35	64	25	29
26	39	74	29	33
31	49	78	40	41

* An experiment, similar to that shown in Table 9 and in Fig. 10 (curves A and D), in which cells were labeled after a period of amino acid starvation, grown for several generations, and then deprived of both thymine and amino acids. Bromouracil was added, and the replication of total and radioactive DNA was measured. In a control, a second portion of the same culture was starved o thymine alone during the second treatment, and replication was again followed. This showed the expected initial selective replication from the chromosome origin, as in Fig. 10 (curve D).

incorporated after a period of amino acid or thymine starvation.

Growth in succinate medium. The experiments just described have been used to gain insight into the replication of chromosomes in cells growing in succinate medium (20). We have shown above that in succinate medium cells contain two chromosomes, only one of which is replicating at any instant. I now would like to examine the order in which these are replicated.

Three possibilities exist: (i) after replicating one of the chromosomes, the other is always replicated next; (ii) after replicating one of the chromosomes, the next chromosome to be replicated is chosen at random from three now present (the two daughter chromosomes just formed plus the one already present); or (iii) after replicating one of the chromosomes, one of the two daughter

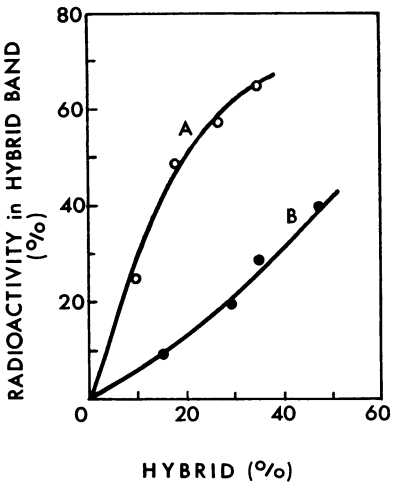

FIG. 11. *Double-label experiment similar to those described in Table 9 and Fig. 10, showing the effect of phenethyl alcohol on chromosome replication (unpublished data). A 20-ml culture of 15T⁻ growing in complete glucose medium (containing amino acids and thymine) at a titer of 2 × 10⁸ cells per milliliter was exposed to 0.25% phenethyl alcohol (PEA) for 90 min. PEA was removed and the culture was incubated for 15 min in medium containing H³-thymine (100 μc/ml, 2 μg/ml). The culture was then diluted to a titer of 2 × 10⁷ cells per milliliter in nonradioactive medium and grown in a volume of 200 ml to a titer of 2 × 10⁸. At this time the culture was divided, and 100 ml was transferred to medium containing 5-bromouracil instead of thymine. Samples were taken at intervals and analyzed for their content of both radioactive and total, light- and hybrid-density DNA. This is curve B. The other portion was starved of amino acids for 80 min and then transferred to medium containing bromouracil and amino acids. Samples of this were also taken at intervals and analyzed (curve A).*

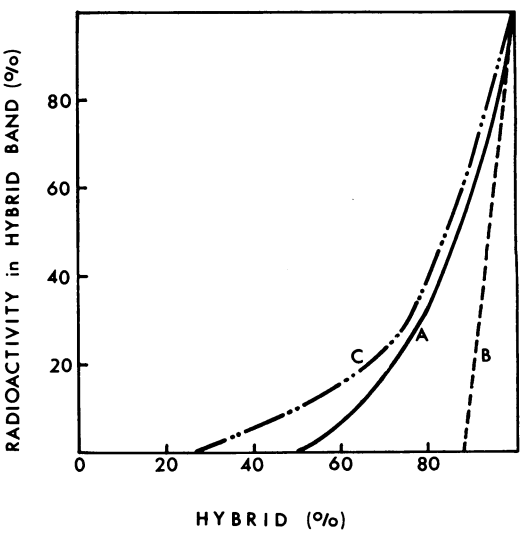

FIG. 12. *Sequential chromosome replication in glucose and succinate cultures (from published data). An exponential culture was pulse-labeled with H³-thymine and then transferred into medium containing 5-bromouracil instead of thymine. (A) Glucose culture (23); (B) theoretical curve expected for the glucose culture; (C) succinate culture (20).*

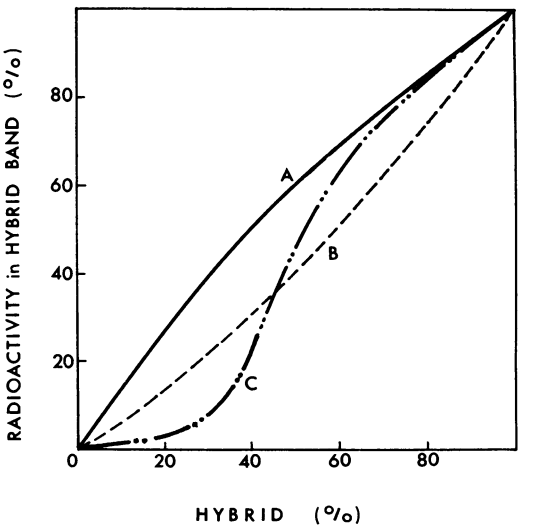

FIG. 13. *Effect of amino acid starvation on sequential chromosome replication in glucose and succinate medium (from published data). Exponential cultures were pulse-labeled with H³-thymine, starved of amino acids in the presence of nonradioactive thymine, and then transferred to medium containing amino acids and 5-bromouracil. (A) Glucose culture (14); (B) theoretical curve predicted for the glucose culture (23); (C) succinate culture (20).*

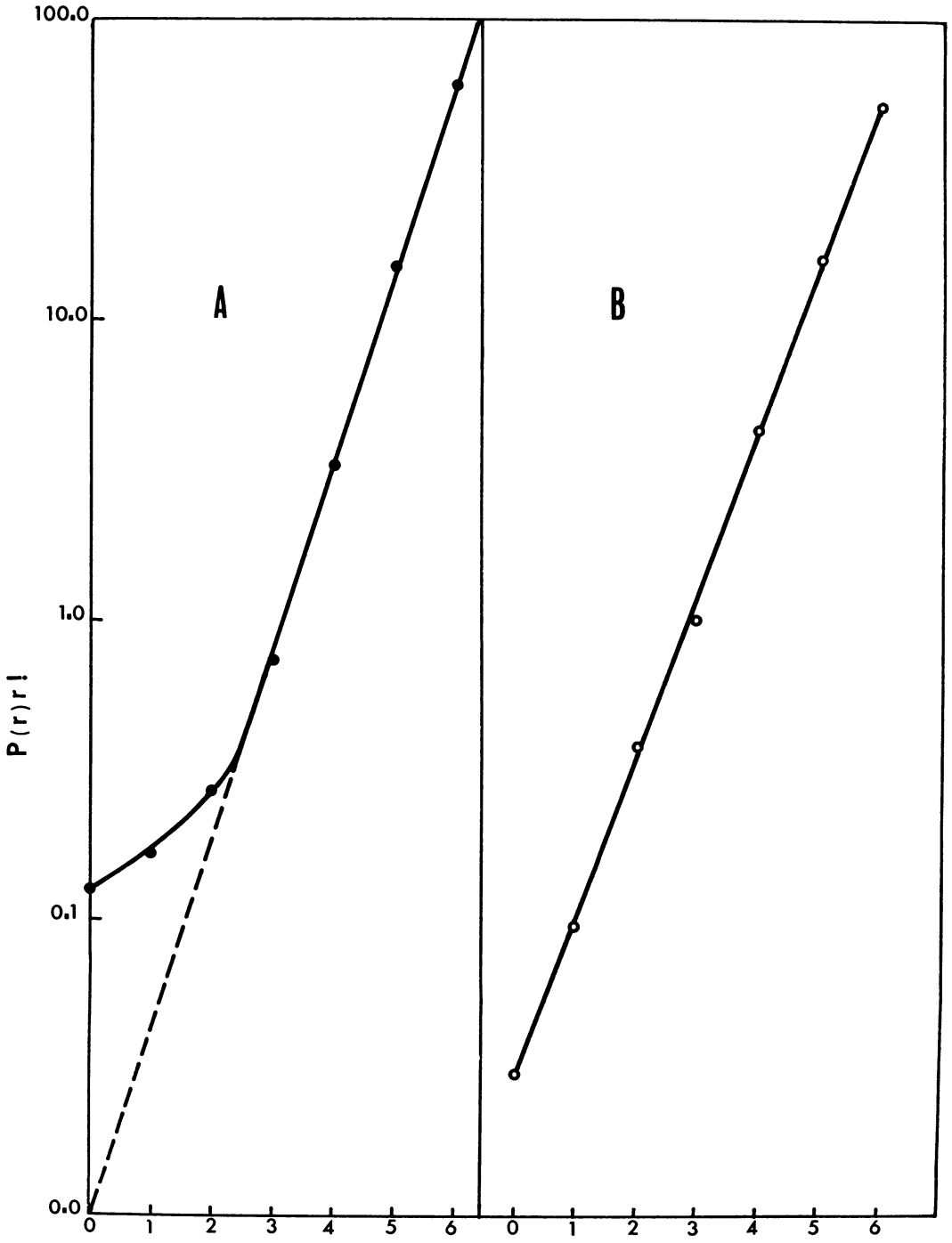

FIG. 14. *Distribution of the number of grains per cell in autoradiographs of cells from a culture which had been pulse-labeled with H^3-thymine 30 min (A) and 50 min (B) after a period of amino acid starvation (unpublished data). The curves are compared to a Poisson distribution: log $[P(r)r!] = r \log n - (n/2.3)$. A detailed description of the experiments is given in Table 11.*

chromosomes just formed is always replicated. If a succinate culture is exposed to a pulse of H^3-thymine and then transferred to medium containing 5-bromouracil, the replication pattern (Fig. 12, curve C) closely resembles that observed in glucose (with 90% of the radioactive material being replicated in a sequential pattern identical to that observed in glucose cultures). This is the pattern of replication expected if a given segment of DNA, having replicated, does not do so again until the rest of the DNA in each cell (which, in this case, includes the other chromosome) has replicated. Further support for this comes from an experiment in which a period of amino acid starvation is interposed between the pulse of H^3-thymine and growth in bromouracil. In contrast to the glucose culture (Fig. 13, curve A), the replication pattern in succinate cultures (Fig. 13, curve C) shows a well-defined lag between the onset of chromosome replication and the replication of radioactive material. This again is to be expected since, in the absence of amino acids, the replicating chromosome will complete its replication cycle but the replication of the second chromosome will not be initiated (Fig. 2 and reference 20). If, when amino acids are restored, the second chromosome must be replicated before the first can begin again, this would exclude the radioactive chromosome from the initial material to be replicated.

Protein Synthesis and the Rate of Chromosome Replication After Amino Acid Starvation

Hanawalt et al. (9) found that after amino acid starvation cells did not appear to commence chromosome replication simultaneously. Instead, they appeared to initiate replication at different times, and all of the cells had commenced chromosome replication within 30 min after the restoration of amino acids. Their observations explained the absence of synchronous cell division after amino acid starvation.

We observed a similar delay in the onset of chromosome replication (23) in experiments of the type described in Fig. 10 and 13. A striking observation was the finding that, after amino acid starvation, DNA synthesis proceeded much more rapidly in some cells than in others. Thus, at the end of 120 min of growth after amino acid starvation, some 30% of the original DNA had not replicated, whereas 20% of the DNA had undergone two cycles of replication (23). The recent discovery that more than 99% of the cells were synthesizing DNA (as determined by radioautographs) leads to the hypothesis: *after amino acid starvation, the rate of replication of that portion of the chromosome synthesized in the absence of amino acids is much lower than the rate of replication of the rest of the chromosome.*

TABLE 11. *Heterogeneity in the rate of DNA replication among cells recovering from amino acid starvation (unpublished data)**

Culture grown in	Time of sample after restoration of amino acids	Length of pulse	Avg no. of grains/cell	Cells not in distribution	Avg no. of grains/cell for cells in distribution
	min	*min*		%	
Glucose	20	6	2.51	55	4.2
	25	6	3.35	37	3.9
	30	6	3.32	30	4.2
	35	6	3.75	10	3.8
	40	6	4.60	5	4.1
	50	5	3.66	0	3.5 (4.2)†
Succinate	20	6	2.01	67	4.0
	25	6	2.14	46	3.75
	30	6	2.25	46	3.6
	35	6	2.42	57	3.85
	40	6	2.28	43	3.6
	50	5	1.72	40	3.0 (3.6)

* At intervals, portions of a glucose or succinate culture recovering from 80 or 90 min of amino acid starvation were given a pulse of H^3-thymine (100 μc/ml; 2 μg/ml). Immediately after the pulse, the samples were washed free from medium radioactivity and analyzed by radioautography. Samples from the glucose culture were exposed for 2 days, samples from the succinate for 4. The radioautographs were analyzed by use of the Poisson distribution, and the slope (average number of grains/radioactive cell) and the percentage of cells not in the distribution were measured. Examples of distributions for the glucose culture are presented in Fig. 14. Upon further exposure (to achieve an average of between 20 and 30 grains per cell), more than 98% of the cells in both the glucose and succinate cultures proved to be radioactive (contain at least 2 grains per cell). Thus, all cells were making DNA.

† The values in parentheses represent the correction for the shorter pulse which these samples received.

The following three experiments establish that this is correct.

The rate of DNA synthesis varies among individual cells during recovery from amino acid starvation. This is shown in Fig. 14 and Table 11, where data are presented from an experiment in which cells recovering from amino acid starvation were exposed to a pulse of H^3-thymine at different intervals after starvation, and the amount of thymine incorporated was measured by autoradiography. If all cells had incorporated the same amount of radioactivity, a Poisson dis-

tribution of grains should have been observed. This is the case for the sample exposed to H^3-thymine 50 min after restoration of amino acids (Fig. 14, curve B). However, up to 40 min after amino acid starvation a distribution was observed in which many cells had incorporated less radioactive material than expected (Table 11). All of these cells were synthesizing DNA as shown by long-exposure autoradiographs. The number of such cells decreased as the period after addition of amino acids increased. In succinate cultures, this heterogeneity was much greater, continuing for more than 50 min after the readdition of amino acids, with almost half of the cells synthesizing DNA at abnormally slow rates. This greater heterogeneity is reflected in the small amount of preferential double labeling observed in succinate cultures after the second period of amino acid starvation in a double-label experiment of the type described in Table 9 (see reference 20).

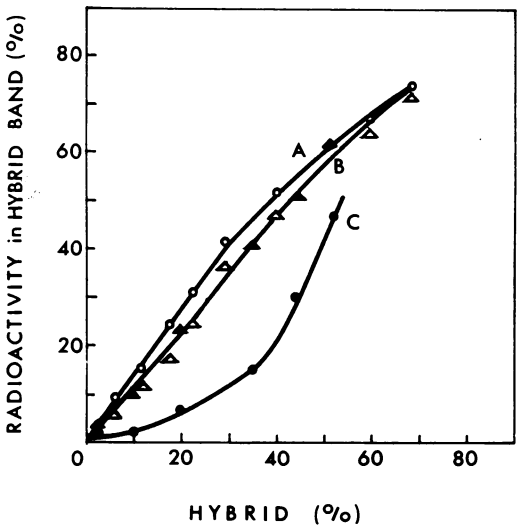

FIG. 15. *Comparison of the subsequent replication of DNA, previously made in the absence of amino acids with the subsequent replication of DNA previously made in the presence of amino acids. An exponential glucose culture (200 ml) of 15T⁻ (2 × 10⁸ cells per milliliter) was labeled with H³-thymine (1.5 μc/ml; 2 μg/ml) for 5 min in the presence of amino acids. The culture was then transferred to medium lacking amino acids but containing C¹⁴-thymine (0.2 μc/ml; 2 μg/ml). After 5 min of incubation with this radioactive material, the culture was transferred to fresh medium containing nonradioactive thymine but no amino acids. After 75 min of further incubation, the cells were transferred to medium containing amino acids and 5-bromouracil instead of thymine. Samples were taken at intervals, and the replicated DNA (H³-labeled, C¹⁴-labeled, and total) was measured. Curve B (▲), H³-labeled DNA; curve C, C¹⁴-labeled DNA. A control experiment was carried out in which an exponential culture was labeled for 5 min with H³-thymine and then for 5 min with C¹⁴-thymine, both times in the presence of amino acids. This culture was then starved of amino acids in the presence of nonradioactive thymine and transferred to medium containing 5-bromouracil and amino acids. Curve A, H³-labeled control culture; curve B (△), C¹⁴-labeled control culture.*

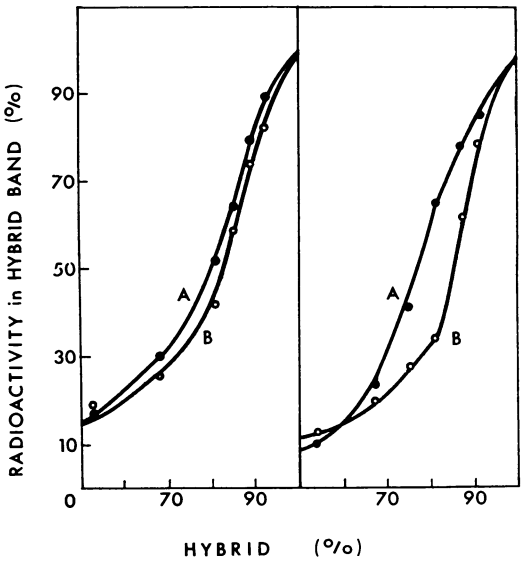

FIG. 16. *Replication of DNA labeled in the presence or absence of amino acids (unpublished data). (right) An exponential glucose culture was labeled (A) with H³-thymine (1.5 μc/ml; 2 μg/ml) for 5 min in the presence of amino acids, and then (B) with C¹⁴-thymine (0.2 μc/ml; 2 μg/ml) for 5 min in the absence of amino acids. It was then transferred to medium containing amino acids and 5-bromouracil. Replication of labeled and total DNA was measured. (left) A control experiment in which both H³ (A) and C¹⁴ (B) label were incorporated in the presence of amino acids.*

Only DNA made in the absence of amino acids is replicated at a slow rate during the first replication cycle following amino acid starvation. This may be seen from the following experiment. A glucose culture is given a short pulse of H^3-thymine, and is then immediately deprived of amino acids and given a short pulse of C^{14}-thymine. The culture is then allowed to complete chromosome replication in the absence of amino acids and in the presence of nonradioactive thymine. Amino acids are restored in the presence of 5-bromouracil, and the replication of the H^3- and C^{14}-labeled DNA is measured (as was done in Fig. 13). The result of such an experiment (curves B and C, Fig. 15) clearly shows that the C^{14}-DNA, made in the absence of amino acids replicates much more slowly than the H^3-DNA,

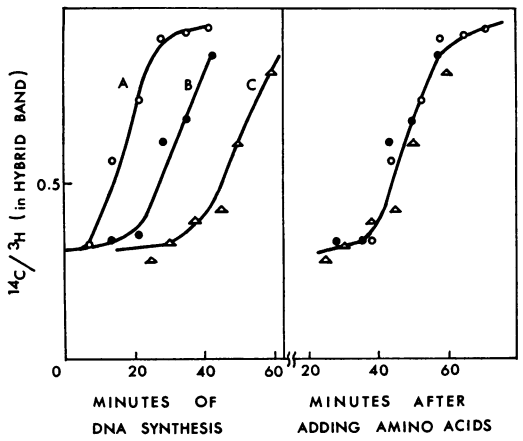

FIG. 17. *Restoration of the replication rate of DNA made in the absence of amino acids (unpublished data). A glucose culture was treated as in the experiment in Fig. 15, except that, after amino acid starvation was completed, the culture was divided into three portions. One was transferred into 5-bromouracil immediately (C); another was incubated for 15 min in the absence of thymine, and then 5-bromouracil was added (B); and the third was incubated for 30 min in the absence of thymine, and then 5-bromouracil was added (A). The replicated C^{14}- and H^{3}-DNA were measured at intervals. The ratios (C^{14}/H^{3}) of replicated DNA are compared (left) as a function of the interval of DNA synthesis (time in 5-bromouracil); (right) as a function of the time after amino acid starvation ended. As replication resumes a normal rate, this ratio approaches 1.*

made immediately before but in the presence of amino acids. Since the two types of radioactivity label adjacent portions of the chromosome, they should show almost identical patterns of replication, as they do when both regions are synthesized in the presence of amino acids (curves A and B, Fig. 15).

The slow rate at which the DNA made in the absence of amino acids subsequently replicates is not a function of its position on the chromosome (e.g., relative to the origin). This is seen from an experiment (Fig. 16) similar to that just described, but in which cells were transferred into medium containing amino acids and bromouracil immediately after labeling with C^{14}-thymine in the absence of amino acids. Now, as shown before (Fig. 12), the labeled regions will be among the last to be replicated. But, as in Fig. 15, replication of the C^{14}-labeled region (DNA made in absence of amino acids) was delayed relative to the H^{3} label and to a much greater extent than in the control experiment (in which both labels were originally inserted in the presence of amino acids).

The rate of replication can be restored to normal if cells are incubated in the presence of amino acids under conditions in which DNA synthesis is prevented but protein synthesis allowed (as in the absence of thymine). This is shown in Fig. 17, where the ratio of replicated C^{14}-DNA to replicated H^{3}-DNA is plotted as a function of the time during which DNA is synthesized or of the time after restoration of amino acids. It can be seen that the recovery of the ability to replicate DNA at a normal rate depends upon the period which has elapsed after amino acid restoration, and is independent of the period of DNA synthesis which has elapsed. The ratio has in all cases returned to normal 60 min after the readdition of amino acids, which is in good agreement with the autoradiographic data in Table 11.

The steep rise of curve C in Fig. 15 would indicate that the restoration of replication rate is completed in about the same time for regions on the chromosome which are near to, and regions which are far from, the chromosome origin.

These observations explain the discrepancy between the observed and the theoretical replication patterns in Fig. 13 (curves A and B). The radioactive material in those experiments is always located before the region of chromosome synthesized in the absence of amino acids. Therefore, it is replicated at a normal rate, whereas the rest of the chromosome is not. This will tend to move the experimental points toward the left on the abscissa. The heterogeneity observed in the experiments in Fig. 10 (curves A, C, and D) may be similarly explained.

These data indicate that the rate of protein synthesis during one cycle of DNA replication may influence the rate of DNA replication during the subsequent replication cycle.

Thymine Starvation and the Initiation of Chromosome Replication

Thymine starvation leads to the initiation of replication from the chromosome origin. This phenomenon is described by the model in Fig. 18. After a period of thymine starvation, chromosome replication resumes at that point on the chromosome where replication ceased and, in addition, a cycle of chromosome replication is prematurely initiated on one of the two paritally completed daughter chromosomes. At the end of the replication cycle, this situation is resolved, probably in the manner shown, and replication subsequently is restricted to one site on each daughter chromosome.

A number of experiments have established this model (18, 31). Curve D, Fig. 10, presented the result of an experiment demonstrating that, following thymine starvation, replication is initiated at the chromosome origin. We have found (31)

that the number of cells in which such a premature replication cycle is induced increases linearly during thymine starvation, reaching a maximum at the end of one generation period, that is, 40 to 50 min for cultures growing in glucose medium and 70 to 80 min for cultures growing in succinate medium (14, 31).

I will not reiterate many of the other experimental arguments for all of the parts of the model in Fig. 18. Those interested may consult Pritchard and Lark (31).

However, the restriction of premature chromosome replication to one of the two daughter chromosome replicas will prove of special importance in our subsequent discussion. Therefore, experiments substantiating this conclusion will be presented.

If an experiment such as that outlined in Table 9 is performed, but in which both treatments of amino acid starvation are replaced by thymine starvation, the replication pattern shown in Fig. 19A is observed. Only 25% of the radioactive label is in the region first replicated. This is most easily explained by assuming that, after the first thymine starvation, half of the label was incorporated at the chromosome origin and the other half at some random location on the chromosome. After several generations of growth, those replicating chromosomes which are labeled at their origin will bear this label in only one of the two available partial chromosome replicas. After the second period of thymine starvation, 5-bromouracil will be incorporated, beginning from half of the available chromosome origins selected at random. This will result in the selective premature replication of only 0.5 by 0.5 or 0.25 of the H^3-thymine-bearing regions of the DNA.

This conclusion is further strengthened by measurements of the amount of H^3-thymine incorporated into the DNA of cultures starved for amino acids, compared with the incorporation into cultures first deprived of thymine and then of amino acids. The result of such experiments (Fig. 20) indicates that almost three times as much radioactivity is incorporated after thymine starvation. This is approximately the amount expected if, in addition to completing the replication of the original chromosome in the absence of amino acids, one extra complete chromosome is synthesized.

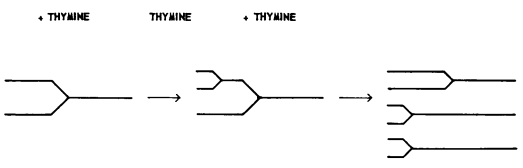

FIG. 18. *Representation of the change in chromosome replication which follows thymine starvation.*

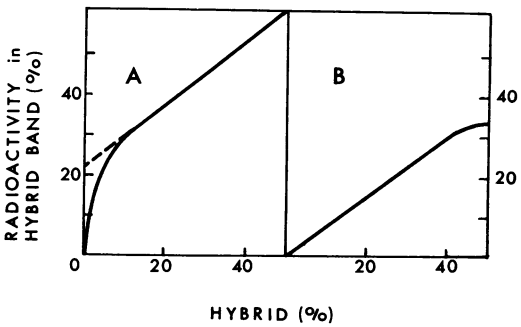

FIG. 19. *(A) Double-label study of the replication of DNA synthesized immediately after thymine starvation (from published data) (31). A glucose culture was starved for thymine, pulse-labeled with H^3-thymine, grown for three generations, again starved for thymine, and finally incubated with bromouracil. The replication of the H^3-labeled DNA was measured. (B) DNA replication after thymine starvation (20). A succinate culture was pulse-labeled with H^3-thymine, starved for thymine, and then placed in 5-bromouracil. The replication of the radioactive DNA was measured.*

Direct proof of the hypothesis has been obtained by radioautography. This experiment was carried out with a succinate culture in which cells contain only one replicating chromosome. At the end of the amino acid starvation period, radioactive cells from an experiment of the type presented in Fig. 20 were placed on agar and allowed to develop into microcolonies. These were examined by autoradiography, and the number of grain clusters in the colonies was scored (18). These grain clusters correspond to the conserved units of DNA which were labeled. The distribution of such grain clusters is shown in Fig. 21. It can be seen that both the normal and the thymine-starved cultures yield unimodal distributions, and that after thymine starvation the mode of the distribution shifts from 2 to 4. It can be concluded that the number of replication points in each cell has doubled as a result of thymine starvation, and it has already been demonstrated that the extra replication point occurs at the chromosome origin.

It can also be demonstrated that the additional replication point occurs on this same chromosome, not the resting one. (Succinate-grown cells contain one replicating and one resting chromosome.) This is seen from the experiment in Fig. 19B, in which a succinate culture was given a pulse of thymine, then starved of thymine, and finally incubated with 5-bromouracil. Radioactivity was immediately observed in material of a hybrid density, indicating that premature replica-

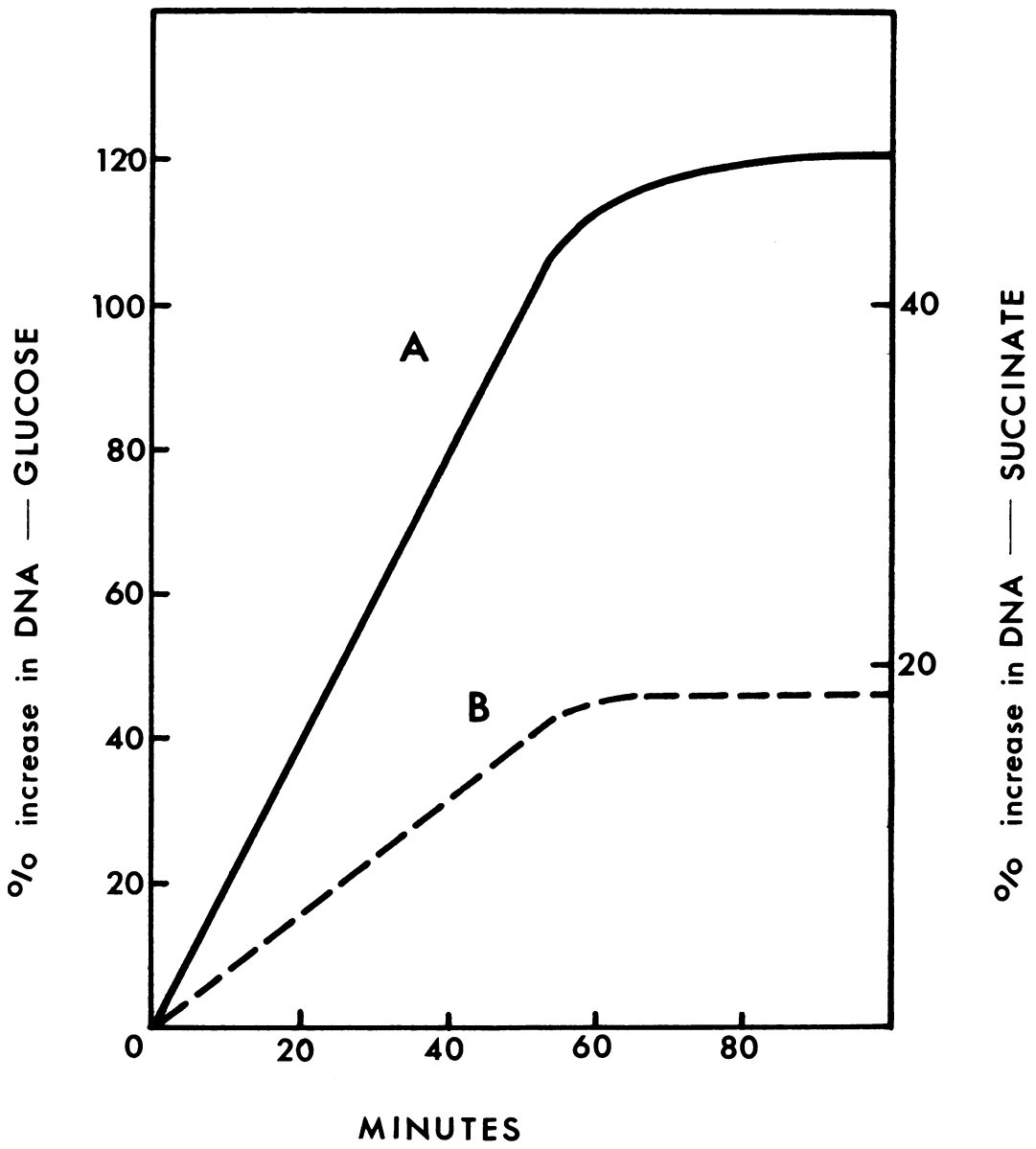

FIG. 20. *Synthesis of DNA in the absence of required amino acids by glucose or succinate cultures (from published data). (A) First starved of thymine, then starved of amino acids; (B) starved only of amino acids. Left hand ordinate = increase in DNA content in a glucose culture (31). Right hand ordinate = increase in DNA content in a succinate culture (20). Abscissa = minutes of incubation without amino acids.*

tion proceeded from the origin of the labeled chromosome (i.e., the one in the process of replicating prior to thymine starvation). This result may be contrasted to the situation occurring in amino acid-starved succinate cultures (Fig. 13, curve C).

Finally, it should be noted that the extra chromosome, which is synthesized as a result of thymine starvation, is stable, and is observed in autoradiography experiments (as in Fig. 21) to be composed of two units which are conserved during subsequent division cycles.

In summary, thymine starvation induces a premature replication cycle which is restricted to

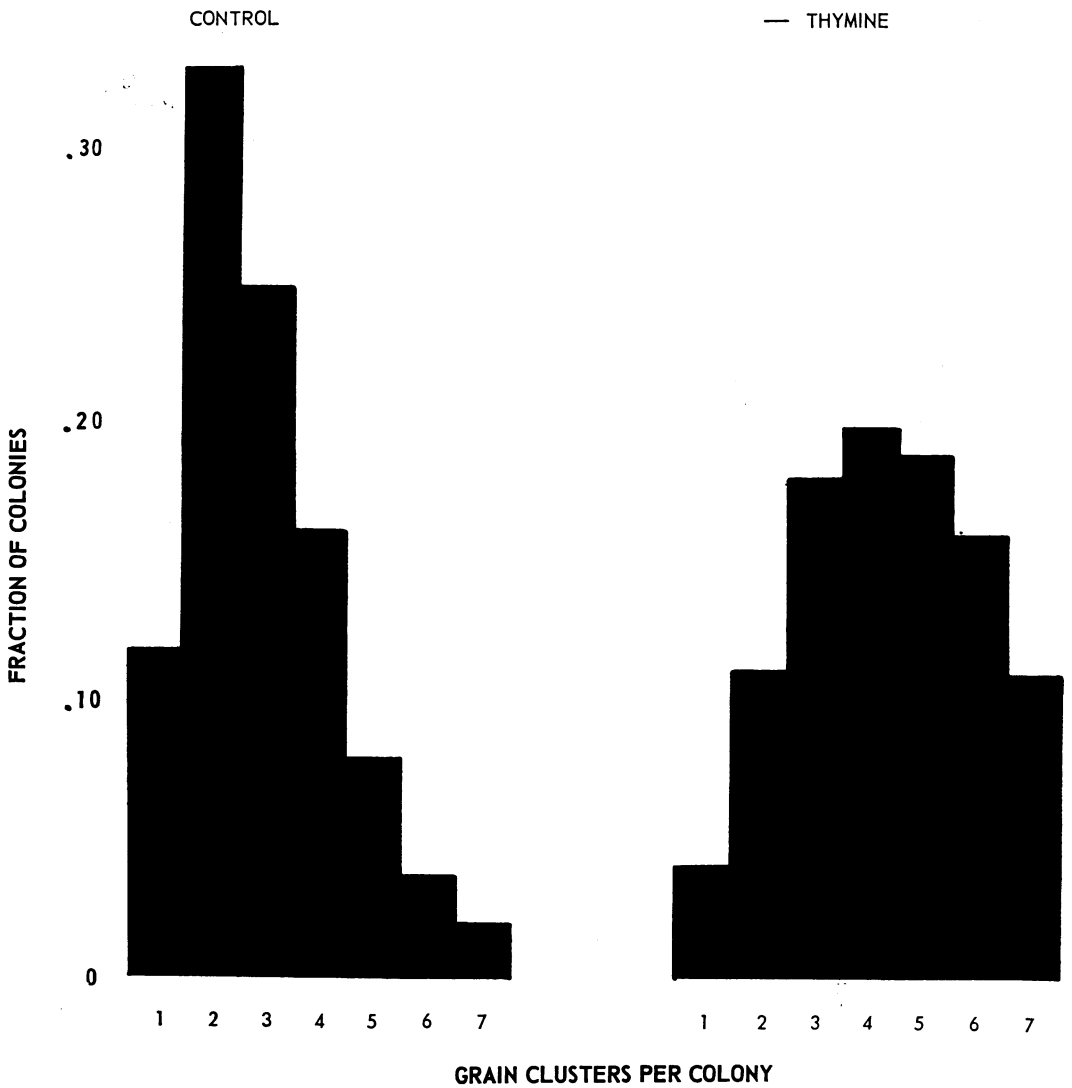

FIG. 21. *Distribution of grain clusters (conserved DNA units) in microcolonies arising from succinate-grown cells labeled with a pulse of H^3-thymine before or after a period of thymine starvation (from published data) (18). A succinate culture was divided into two portions. One was starved of thymine; the other was not. Both were pulse-labeled with H^3-thymine. Cells from both were allowed to grow into microcolonies of 32 to 64 cells, and autoradiographs were prepared. The number of grain clusters in each microcolony was determined.*

one of the two partially completed progeny of those chromosomes already in the process of replication.

Re-initiation Versus Premature Initiation of Chromosome Replication

After amino acid starvation, replication proceeds from the chromosome origin only. This we have called re-initiation of replication. After thymine starvation, on the other hand, replication proceeds from the origin in addition to that point on the chromosome where replication was interrupted by starvation. This we have termed the premature initiation of replication. The two processes are not identical.

The data in Table 10 demonstrate that amino acid starvation during thymine deprivation will block premature initiation of replication. Restoration of amino acids, of course, is necessary for reinitiation of replication. Thus, both processes

appear to involve the synthesis of protein. However, the types of protein involved are different.

After amino acid starvation, chromosome replication may be reinitiated by the addition of amino acids in the presence of either chloramphenicol or 5-fluorouracil (14). On the other hand, the premature initiation of chromosome replication may be completely blocked if either of these agents is present during thymine starvation. The inhibitory effect of fluorouracil could be reversed by the addition of an excess of uracil. The experiments supporting these conclusions were of the same type as those presented in Table 9 and Fig. 10 (curve A or D). If, in an experiment of the type described by the protocol in Table 9, amino acids were restored in medium containing 5-bromouracil in the presence of either chloramphenicol or 5-fluorouracil, the replication of labeled DNA followed a pattern similar to curve A in Fig. 10, although the rate of chromosome replication was decreased. On the other hand, in experiments in which thymine starvation was substituted for the second period of amino acid starvation, the presence of either chloramphenicol or 5-fluorouracil during thymine starvation altered the subsequent replication pattern from that of Fig. 10, curve D, to that of curve B. Uracil, when present with fluorouracil, restored the pattern to that of curve D.

Although chloramphenicol did not prevent reinitiation of replication, it reduced the initial rate and the total amount of replication to about half that normally observed.

These data indicate that there are two distinguishable processes which may initiate chromosome replication. One is involved in premature chromosome replication, whereas the other or perhaps both may be involved in reinitiating replication. The data exclude the possibility that only a single step, i.e., the synthesis of one type of protein, is involved in both processes.

DISCUSSION

The chromosome replication patterns summarized on page 8 are characterized by three parameters: the chromosome content of the cell; the time at which replication of a chromosome is initiated; and the rate at which replication progresses along the chromosome, once initiated.

Rate of Chromosome Replication

At present, our information concerning the rate at which replication progresses is meager. There can be little doubt, in view of the evidence now available, that replication proceeds sequentially from the beginning to the end of the chromosome. The assumption that replication proceeds at a linear rate along the entire chromosome has not been tested in our experiments, but they do not contradict it. Cairn's (6) radioautographic studies would indicate that this assumption is correct, since he observed that in almost all of the cells of a glucose culture a constant linear stretch of chromosome was synthesized during a short time interval, i.e., 10% of the generation period.

The data summarized on page 8 clearly demonstrate that, once initiated, replication may proceed at different rates in different growth media. This may be related to the finding that DNA which has replicated in the absence of amino acids is subsequently slow to replicate (Fig. 16 and 17). In that instance, it was found that permitting protein synthesis for almost an entire generation period was necessary to restore the rate of DNA synthesis to normal. A longer period seems to be required by cells growing at a lower rate in succinate medium (Table 11), and still longer periods may be required in media in which growth is even slower. Thus, the time elapsing between the first and the second replication of a given portion of a chromosome may not be sufficient to allow that protein synthesis necessary to effect those changes in the chromosome which result in rapid replication. In this case, when chromosome replication is initiated, replication of portions of the chromosome may proceed at a slow rate because a certain amount of this essential protein has not been produced.

Chromosome Content and the Initiation of Replication

My objective in this section will be to present a model for chromosome replication which explains the patterns of replication and segregation observed. In the course of its synthesis, several other models were discarded because they conflicted with one or another aspect of the data. These will not be discussed. The model presented differs in some respects from that which we proposed previously (14, 20), but affords a better explanation of the available facts. In addition, in its more general aspects it can be applied to the replication and segregation of the chromosomes of higher organisms. I will eventually relate it to the replicon model proposed by Jacob, Brenner, and Cuzin (11).

I will begin by discussing the observed patterns of segregation (Fig. 6 and 7). These results demonstrated that each of the chromosomes derived from two parent chromosomes was distributed into daughter cells in such a manner as to bring together into one daughter cell conserved units of DNA, one of which had been

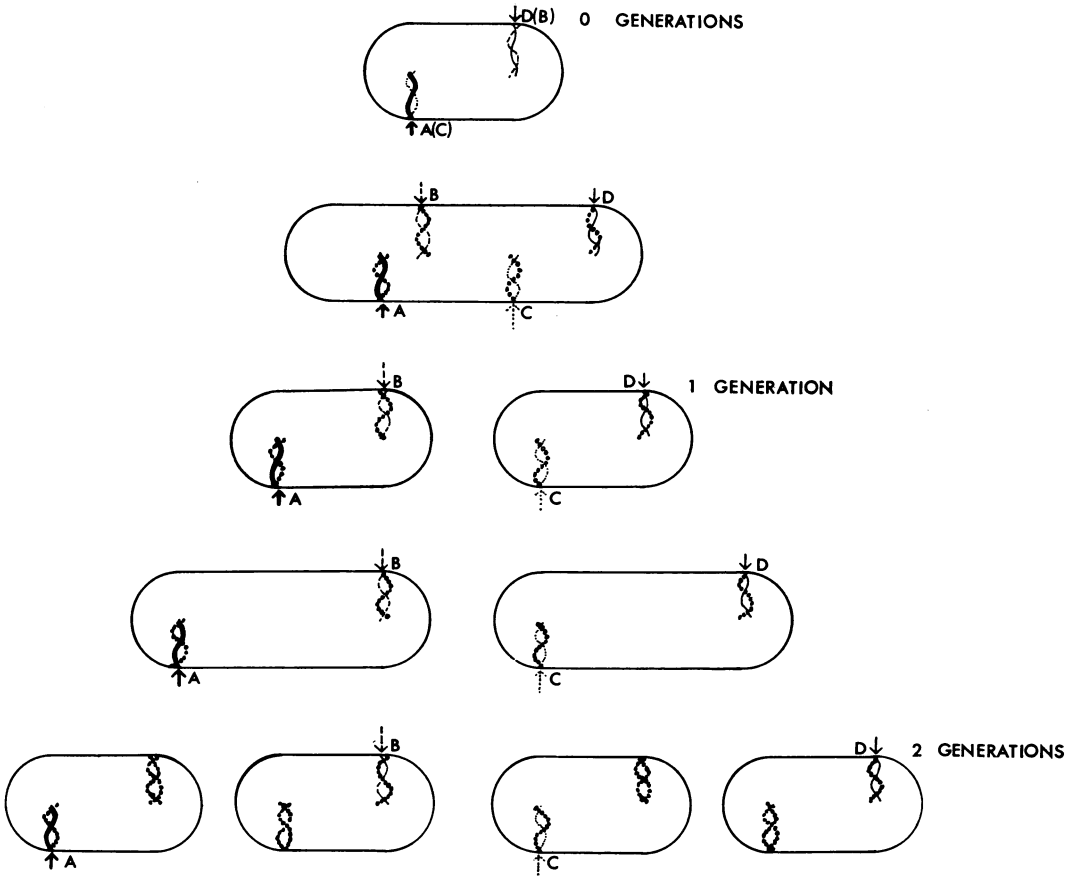

FIG. 22. *Segregation of conserved units of DNA, such that every cell receives one such unit in the second generation. Two chromosomes, containing four conserved units of DNA (A, B, C, and D), are present at the beginning of replication. One conserved unit of each chromosome is permanently attached to the cell surface (i.e., A and D). The other two are unattached, represented by parentheses. Upon replication, the conserved units separate as shown, and B and C also attach permanently to the cell surface. Repetition of this process separates the four units into the four cells which have formed by the second generation. This process distinguishes conserved units used as templates for the first time, (C) and (B), from conserved units being used as templates for the second time, A and D. At each division, a daughter cell receives two chromosomes, one formed from each type of template.*

available as a template only in the previous round of replication (parent) and one of which had been used as a template in the previous two rounds of replication (grandparent). A model for such segregation is presented in Fig. 22. Each chromosome is assumed to be fixed to the membrane-wall complex by one of its two component strands, whose polarity is distinguishable as either a 3′- or a 5′-ended polynucleotide. In replicating, the other template strand (5′- or 3′-ended respectively) becomes fixed to the wall-membrane complex in another position, which will eventually distribute it into a daughter cell. A similar process occurs for the second chromosome. When replication is completed, the progeny chromosomes separate, assuming their new positions in the cell or retaining their old position depending upon which chromosome strand was acting as a template. At this stage all of the chromosomes are now fixed to the wall-membrane complex by those strands which serve as templates in the process of replication. The process is again repeated in the next cycle, with the result that the four original conserved units of DNA are eventually distributed into four different cells. Two are never distributed into the same cell. The basic principle on which this distribution is based is that, in the process of serving as a template for replication, a conserved unit of DNA becomes fixed into a permanent position on the membrane-wall complex. Thus, such a *fixed single DNA strand is the basic unit of chromosome segregation.*

This fixation implies that a position exists to which attachment occurs. If we assume that this position and attachment is ultimately designated by a process involving protein synthesis, and that attachment is a requisite first step in replication, we can easily understand that depriving cells of required amino acids will lead to a cessation of replication after the completion of the replication cycle which is already underway. At this stage in our model, then, replication occurs in the following steps: the existence of a chromosome fixed by one strand to the cell wall-membrane complex, the subsequent attachment of the other conserved unit to initiate replication, sequential replication, and separation of the chromosomes. The cycle then repeats.

It is easy to see how a reduction in the growth rate may also reduce the rate of production of positions to which the chromosome may attach. As long as the reduction is proportional to the reduction in the division rate, the number of chromosomes per cell will remain constant. However, when such positions are created at a rate which is less than the division rate, the number of chromosomes per cell will be reduced. Thus, in glucose or succinate media two new places of attachment may be created during one division cycle, maintaining two chromosomes per cell. In aspartate, proline, or acetate media, only one would be synthesized per division cycle, reducing the chromosome content per cell to one. As the growth rate becomes slower, the synthesis of new sites also becomes slower, until, after each division, there is a lag in chromosome replication while synthesis of the attachment site is completed (as in proline or acetate cultures).

According to this model, cells growing in succinate medium, although making sufficient attachment sites to maintain two chromosomes per cell, make them at a much slower rate than in glucose medium, with the result that only one is produced during half of a generation period.

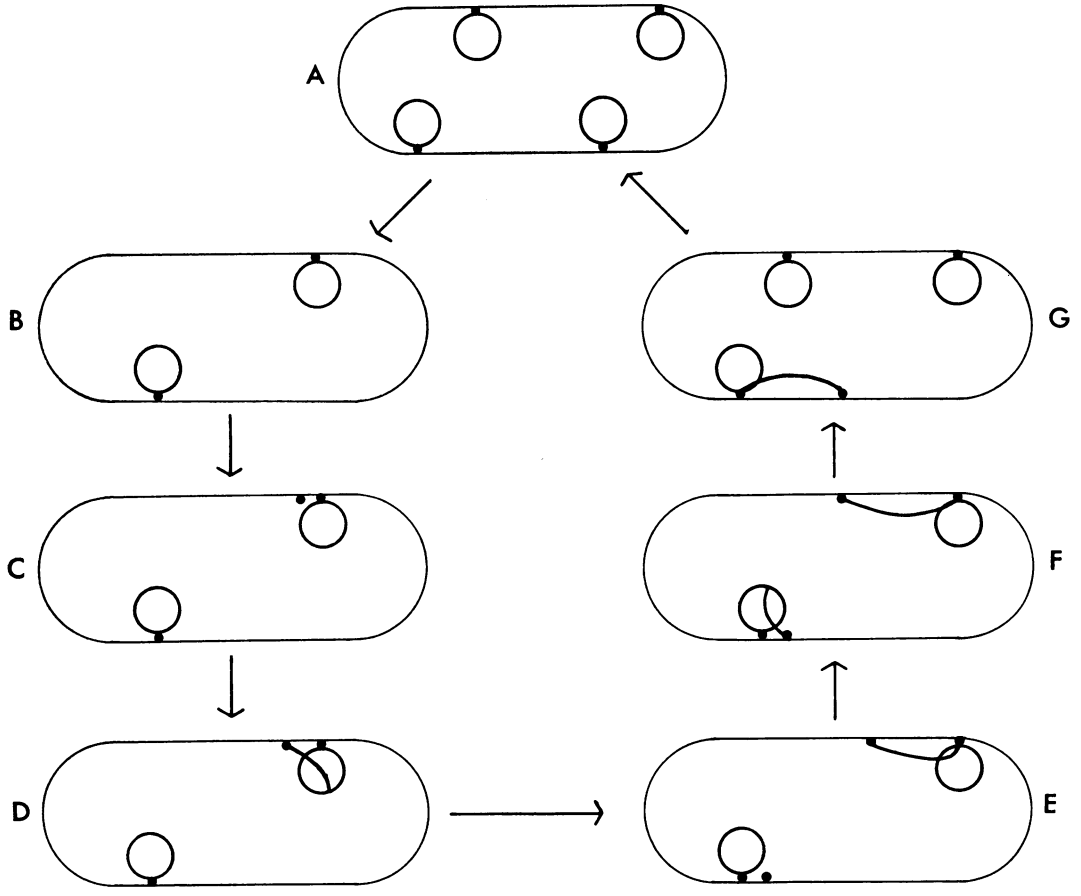

FIG. 23. *Possible mechanism for the alternating chromosome replication observed in succinate cultures. The point of attachment to the cell surface is represented as* ●.

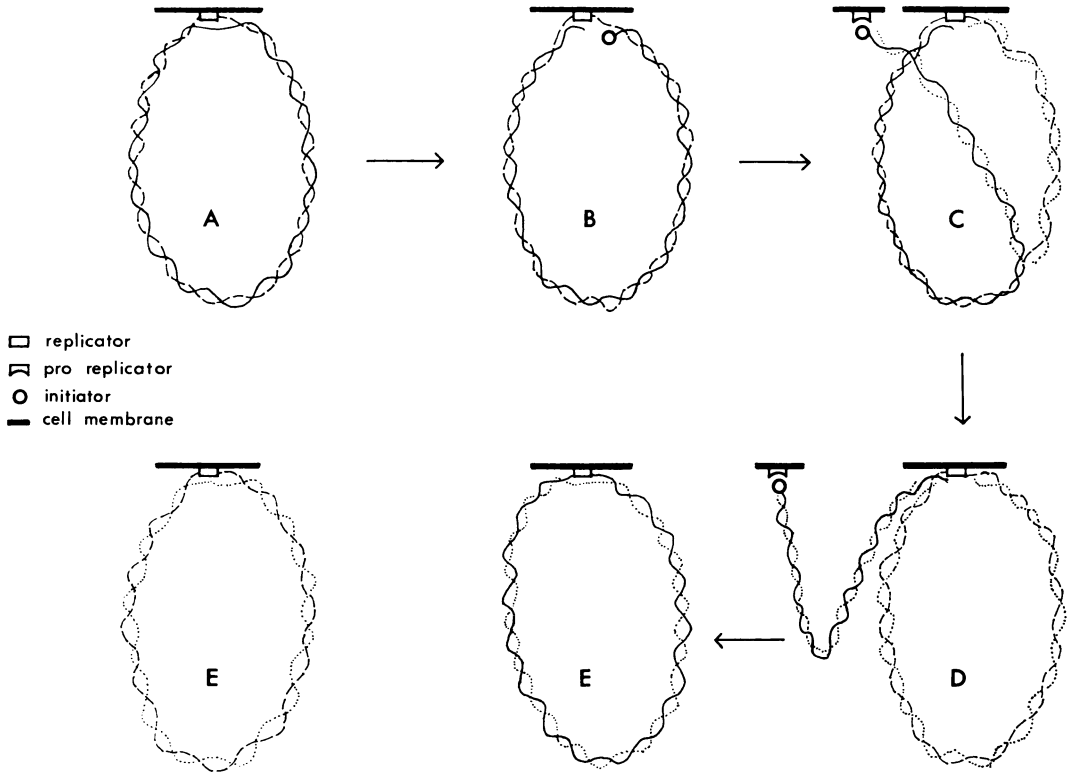

FIG. 24. *Replication of a chromosome in Escherichia coli. One strand of the chromosome is permanently attached to the cell membrane by a protein, the replicator. The other is detached by interaction with the initiator, and is temporarily attached to the cell membrane via a protein, the proreplicator. Permanent attachment of this strand results when replication is completed, thus converting the proreplicator into a replicator.*

This would restrict replication to one chromosome at any time. The alternating replication observed suggests two possibilities. (i) Synthesis of new attachment loci is regulated in such a manner that, if a new site suitably located for the attachment of one chromosome is made, the site for the attachment of the other is always completed next. (ii) The separation of the progeny of a replicated chromosome is not completed until after the other has begun to replicate.

According to this second alternative (Fig. 23), a cell with two chromosomes would begin the replication of one as soon as both of its conserved units were fixed to two positions. The second chromosome, however, would be unable to replicate until another site for attachment was available. This second site would become available prior to the separation of the two progeny of the first chromosome, thus excluding their use of the site.

The number of sites which are available for attachment should not increase instantly after a shift from succinate to glucose. Instead, about one glucose generation period would be necessary to allow their production. Thus, the succinate replication pattern (one chromosome replicating at any time) will persist until most of the cells have completed the chromosome which is in the process of replicating. Similarly, after a shift from glucose to succinate, the reduction in rate of synthesis of the attachment sites will not become apparent until both chromosomes have completed replication, and only one site is available for the reinitiation of a cycle of replication.

Another point of interest is that the segregation model in Fig. 22 is based on the regular replication and distribution of chromosomes into daughter cells. If an extra chromosome is introduced, the geometry of the cell may become confused and the segregation pattern could be disrupted. This is the case in cells which receive a chromosome during mating and in cells after thymine starvation. We have followed the segregation of radioactive chromosomes synthesized by a succinate culture which had been starved of thymine. The segregation pattern does not correspond to the pattern either in Fig. 6B or 7B or to the glucose-to-succinate data in Table 6. Rather, it corre-

sponds to a combination of the pattern expected for succinate cells (i.e., Fig. 6B), together with the random distribution of two additional conserved units of DNA into daughter cells. Thus, it may be difficult to interpret experiments based on the segregation of chromosomes introduced into the cell by techniques other than natural replication.

Finally, it should be noted that the succinate segregation pattern is the result of adequate separation of chromosomes prior to division. If this did not occur, daughter chromosomes would remain in the same cell at each division, resulting in a pattern similar to that expected from a binucleate cell (in which the chromosome pairs A, C or B, D in Fig. 22 would each be isolated in a daughter cell). This may occur at faster growth rates.

Let us now take a closer look at the initiation of replication and the nature of the attachment of the chromosome to the cell wall. The basic model for this is shown in Fig. 24. The chromosome is a ring, one strand of which is attached by a protein (replicator) to the cell wall-membrane complex. The other strand must be broken (as proposed by others for the replication of viral nucleic acid) in order for replication to proceed. This broken strand becomes temporarily attached via protein (the initiator) to a site (proreplicator) possibly on the membrane, thus opening a stretch of the double helix and initiating replication. As replication proceeds, two things occur: the proreplicator is located at its ultimate site on the cell surface, and the helix unwinds, rotation occurring around the single phosphodiester bonds of the strand attached to the replicator. When replication is complete, the ends of the chromosome attached to the initiator rejoin at the proreplicator, converting it into a replicator, and the initiator may be liberated for possible reuse. The reopening of the unattached strand and its attachment to another initiator repeats the process.

The following subsidiary provisions are of interest, although they are not essential to the model. The fixation of the chromosome to the cell surface implies the permanent closure of the ring of one strand but the temporary closure of the other. Temporary closure can be brought about if there is a complementary redundancy between the nucleotide sequence at the beginning and at the end of one strand of the chromosome, an accomplishment easily brought about by replicating past the point of origin on the ring. This situation would be similar to the cohesive closure of the lambda chromosome (10, 35). Permanent closure would be brought about if such complementary redundancy in the nucleotide sequence served as a signal for the formation

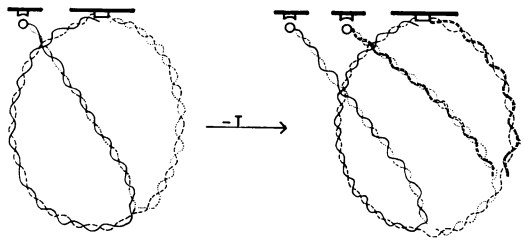

FIG. 25. *Extension of the model in Fig. 25 to explain the premature induction of chromosome replication induced by thymine starvation.*

of a phosphodiester linkage between the ends with the excission of the redundant part, an operation for which a mechanism is already known (30, 34).

It will be seen that one of the basic properties of the chromosome attachment is that it allows one chromosome strand to rotate while remaining fixed to the cell surface, a property which is necessary for the replication of a ring chromosome (5). The rate of formation of replicators will limit the rate at which chromosomes can initiate replication, as will the formation of initiators. A blocking of the former will result in the cessation of chromosome replication. Blocking the latter will restrict replication to a linear rate. Under conditions of thymine starvation, in which chromosome replication ceases, the conversion of proreplicators into replicators will also cease, since this depends upon the completion and separation of the chromosome. On the other hand, the formation of more initiator protein and, possibly, proreplicator sites initiates the replication of the newly synthesized free strand complementary to the strand attached to the replicator. Since the polarity of both strands attached to initiators is the same (Fig. 25), the rotational demands on the replicator will be the same, and two new chromosomes can be synthesized by use of a single replicator-attached strand as a template. The model thus restricts the initiation of an extra replication site to the segregation unit, i.e., that strand of the chromosome which is attached to the replicator.

Since it takes twice as long to induce premature replication in a succinate culture as in a glucose culture, it seems reasonable to assume that the rate of formation of the initiator, and of the proreplicator site to which it attaches, is proportional to the rate of cell growth. This is consistent with the dependence of the rate of formation of attachment sites on growth rate, which, as we have already discussed, will tend to limit the rate at which chromosomes will initiate replication and eventually the chromosome content of the cell.

In the absence of required amino acids, replication can proceed, but the replicated chromosomes cannot separate; thus, the proreplicator will not be converted into a replicator.

When amino acids are added back, the proreplicator will be converted into a replicator, and the initiator will be liberated for reuse if required. In the model, chloramphenicol or fluorouracil, while inhibiting the formation of new initiator, will not inhibit the conversion of a proreplicator into a replicator, as they do not inhibit formation of certain types of ribosomal protein (1, 28). Reinitiation, by the pre-existent initiator, is therefore possible in the presence of these inhibitors. On the other hand, further initiator synthesis will be blocked by chloramphenicol or 5-fluorouracil, which thus also prevent premature initiation.

It is clear that the initiator and proreplicator which I have described correspond to the initiator and replicator proposed by Jacob et al. (11). The model presented here corresponds with and extends theirs.

An important aspect of the model is the regulation of chromosome replication by two systems: the rate of cell growth, which governs the synthesis of initiator and proreplicator sites; and the completion of chromosome replication, which regulates the conversion of a proreplicator site into a replicator site.

It is tempting to believe that such a conversion in some way affects the membrane so that, unless it is completed, cell division cannot occur. This would insure that no cell divides before replication and the separation of the chromosomes is complete.

This model may be useful for explaining the observed separation of the radioactive conserved halves of animal cell chromosomes from their nonradioactive counterparts. In such an extension, certain parallels may be found between the centrioles, spindle attachments, etc., and the replicator site described here.

I have presented a model for chromosome replication based on our recent experiments on chromosome segregation in *E. coli* 15T⁻. The essential feature of the model is that the chromosome is permanently attached to the cell surface by the basic unit of segregation, which is a single strand of the DNA double helix, one of the two conserved units which compose the chromosome. The nature of this attachment preserves a distinction between the newest and oldest template strands, and between them and their newly formed complements. Replication and segregation are thereby arranged in a manner conforming to the restriction of this attachment.

ACKNOWLEDGMENTS

The ideas and concepts presented evolved during continual discussions with my wife, Cynthia, whose experimental contribution is already evident from the bibliography. This collaboration added another dimension of enjoyment to the work.

I thank Rollin Hotchkiss for his patient and understanding help with the manuscript. His skill as a midwife and plastic surgeon made the worthwhile parts of this manuscript possible. This task was aided by the excellent help of Sharlene Gardner, whose ability to meet various deadlines transformed vague scribbling into legible reality.

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