AUTORADIOGRAPHIC STUDIES OF BACTERIAL CHROMOSOME REPLICATION IN AMINO-ACID DEFICIENT *ESCHERICHIA COLI* 15T⁻

FREDERICK FORRO, JR.
From the Department of Molecular Biophysics, Yale University, New Haven

ABSTRACT Autoradiographic experiments on amino-acid requiring strains of *Escherichia coli* T⁻ have been performed with fully-labeled cells harvested in log-phase and after periods of amino-acid starvation. The simplest segregation of incorporated label among progeny grown on non-radioactive medium is into two packets. The result corroborates the two-unit model of *E. coli* DNA inferred from previous studies with partially labeled cells. Following amino-acid starvation, the distribution of label among clones derived from labeled cells indicates cells are grouped into classes having DNA contents in the ratios 1:2:4. The segregation of label among progeny isolated by micromanipulation from such starved cells supports the view that the chromosomes are brought to a state of completed synthesis with different cell classes containing different integral numbers of chromosomes. Infrequent clones interpretable as arising from cells with three chromosomes suggest that the control of replication of chromosomes lying in the same cytoplasm is on an individual basis. The block in DNA synthesis resulting from amino-acid starvation is not perfect. Nevertheless, such starvation permits characterization of the dispersive replication events for more homogeneously labeled and definable DNA units than otherwise possible. The size-frequency distribution of label among progeny following six rounds of chromosome replication is close to that expected from a model of random breakage of linear polymers. The frequency of dispersive events is estimated as 0.5 to 0.7 per chromosome per generation and is only slightly influenced by tritium decays occurring during bacterial growth.

INTRODUCTION

The presence in *Escherichia coli* of large DNA-containing structures, possibly single Watson-Crick molecules (1), which replicate semiconservatively has been inferred from the comparison of previous autoradiographic experiments on whole

cells (2-4) and density gradient experiments on extracted molecules (5). Strong support for such DNA organization has been provided by Cairn's demonstration that long circular DNA structures interpreted as replicating semiconservatively are released from *E. coli* by gentle lysis (6, 7). Some justification for identifying these structures as the chromosomes of bacterial genetics can be made (8) and we shall therefore refer to them as bacterial chromosomes.

Although the subunits of the chromosomes tend to be conserved in replication, they are often subject to some process, previously called fragmentation, which leads to sharing of label between daughters (3). Continuous DNA synthesis (9, 10) and asynchrony of replication cycles of log-phase cells growing under conditions of our previous experiments complicates characterization of this fragmentation and other features of the replication process. It would be desirable to examine the segregation of label from a population of cells each containing the same number of completed chromosomes. Ambiguities in the assessment of label distribution arising from the segregation of a heterogeneous collection of partially labeled replicas might thus be lessened. It was therefore encouraging to learn of the behavior of *E. coli* $15T^-A^-U^-$ when grown in thymine but lacking arginine and uracil (11, 12). Evidence was presented which supported the thesis that cells incubated under such nutritional conditions completed only the round of DNA synthesis in progress at the start of arginine and uracil deprivation. Confirmation of this interpretation has come more recently from the experiments of Lark and co-workers (13-15). The results of Hanawalt *et al.* (12) suggested that all cells in the population arrived at a state in which each contained two completed daughter chromosomes. This paper examines these conclusions in greater detail and presents data which support the notion of organization of bacterial DNA in the form of duplex chromosomes, bear on the relation of chromosomal replication to the bacterial division cycle, and characterize the fragmentation process.

MATERIALS AND METHODS

Bacterial Strains. *Escherichia coli,* strains $15T^-$ (thymine requiring), $15T^-A^-U^-$ (thymine, arginine, and uracil requiring), and $15T^-H^-$ (thymine and histidine requiring) were gifts of Dr. Seymour Cohen.

Bacterial Growth. Liquid culture medium was either the mineral medium M-9 or C as described by Roberts (16) with glucose, usually 0.16 per cent (w/v) but sometimes 0.4 per cent, as the carbon source. Supplements as required included thymidine 4 γ/ml, or thymine 2 γ/ml, arginine 20 γ/ml, and uridine 20 γ/ml, or uracil 10 γ/ml. Growth in liquid was performed at 37° ± 1°C. Inocula used in experiments were taken from 1 ml overnight log-phase cultures at cell concentrations $\leq 2 \times 10^8$/ml. Labeling was performed in culture volumes of 0.1 to 0.4 ml with final titers $\leq 2 \times 10^8$/ml. Such titer restrictions and the use of relatively large surface to volume ratios were considered to yield aerated cells.

Growth on agar medium for subsequent autoradiography has been described previously (2). In all experiments reported here, such growth was at room temperature of

23°C ± 1°C, controlled by a thermostated air conditioner. Cell concentrations were determined by counting in a Petroff-Hausser chamber.

Radioactive Labeling. H^3-thymidine was obtained from Schwarz Laboratories, Inc., Mt. Vernon, New York, and H^3-thymine from New England Nuclear Corporation, Boston, Massachusetts. Previous experience had indicated variable amounts of radioactivity in such sources which would distribute among progeny of labeled cells in a random manner (3). Paper chromatography in isopropanol, water, ammonia (70:25:5) consistently revealed a small peak (~5 per cent of the thymidine or thymine) which trailed the main radioactive component. Use of the eluted main peak for labeling and subsequent progeny clone autoradiography indicated that chromatography had eliminated the source of excessive randomizing label. All radioactive stocks were purified by such chromatography and elution.

Different specific activities for labeling were achieved by using variable proportions of radioactive stock and unlabeled nucleoside or base. Precise reduction in specific activity from stock values was difficult to assure with the small culture volumes and concommitantly small volumes of radioactive stock used. Thus, for experiments of similar design, there was some variation among experiments in autoradiographic grain density for a given film exposure.

Removal of radioactivity or organic supplements from cells subsequently to be incubated without these ingredients was accomplished by chilling the culture in an ice bath, followed by three rounds of sedimentation in a microcentrifuge (Misco Co., Berkeley, California) and resuspension in chilled minimal medium. Final resuspension was in medium containing glucose and other desired supplements. Return of chilled washed cultures to a 37°C water bath resulted in temperature equilibration within the culture in approximately one minute.

Measurements of radioactivity were performed with gas-flow Geiger counters calibrated by tritium standards. Preparation of samples used in incorporation experiments has been described elsewhere (4, 17).

Micromanipulation and Autoradiography. These procedures were the same as previously described (3). Where clusters of grains were difficult to resolve, a minimum estimate of the number constituting the cluster was recorded.

Enzyme Digestion. Some coverslips were digested with DNAse (1x crystallized, Worthington, 100 μg/ml in 0.2 M maleate, 0.2 M tris, 0.01 M $MgSO_4$, pH 7.0) prior to autoradiography. The buffer solution alone was used as a control. Warmed solutions were pipetted onto warmed coverslips and incubated at 37°C for 2 hours. The coverslips were then rinsed several times with water and dried.

RESULTS

Properties of E. coli 15T⁻A⁻U⁻. The broad features of incubation with thymine but lacking arginine and uracil (+T−AU) were similar to those described by Maaløe and Hanawalt (11). Cells became protected against subsequent thymineless death, and DNA synthesis as measured by uptake of H^3-thymine decreased steadily to low levels compared with controls (Fig. 1). The kinetics of these events were slower than described by Maaløe and Hanawalt, possibly related to our method of harvesting cells by centrifugation.

Previous autoradiographic study of log-phase cells (3) in minimal-glucose

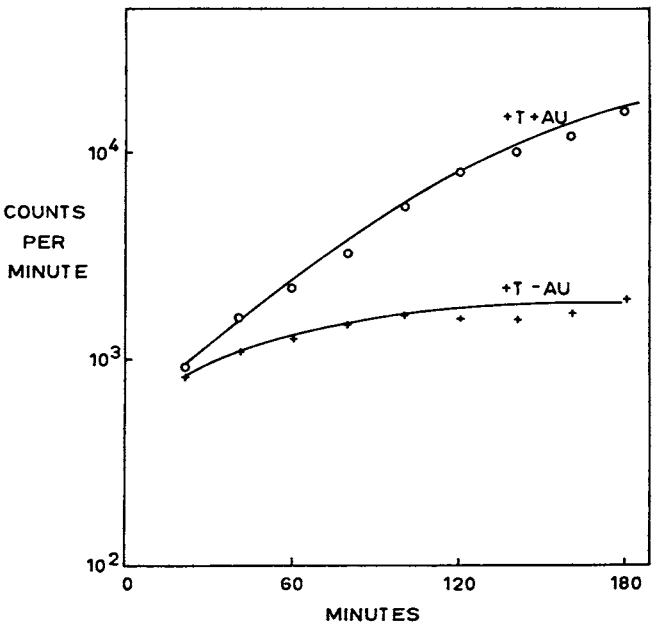


FIGURE 1 Uptake of H^3-thymidine by *E. coli* $15T^-A^-U^-$ grown with and without added arginine and uridine at initial titers of about 1×10^8/ml.

medium led to the view that completion of chromosome synthesis and subsequent cell division were not necessarily closely correlated in time. The data were consistent with the notion that cells containing daughter chromosomes from one round of replication did not divide until each of these chromosomes was substantially advanced on the next round of synthesis. The youngest cells in such a population were considered to contain a single replicating chromosome well along in its cycle of replication. Attempting to amalgamate these ideas with those of Maaløe and Hanawalt, it seemed reasonable to expect that starvation of *E. coli* $15T^-A^-U^-$ for arginine and uracil would bring young cells to the stage of two completed chromosomes. Older (and generally longer) cells might be expected to reach four completed chromosomes. If these older cells divided during such starvation, one might hope that all cells in the population would contain two complete chromosomes. A preliminary experiment based on these thoughts indicated the need for closer examination. Unlabeled log-phase cells were transferred into medium containing H^3-thymine and lacking arginine and uracil and incubated for 150 minutes at 37°C. After washing, cells of different sizes were isolated by micromanipulation and grown to microcolonies. It had been expected that the shortest selected cells would yield colonies containing more label than longer ones since according to the Maaløe-Hanawalt model they would be presumably

further from the completion of their chromosomes at the start of labeling. Surprisingly, some of the smaller cells gave colonies with very little label, whereas others were heavily labeled. This led to the conjecture that such short cells might have arisen by division from cells which had completed chromosomal replication shortly after the start of starvation and that the population might not be homogeneous with respect to DNA content per cell. It was decided, therefore, to re-examine this question of population homogeneity.

Cells were fully labeled by log-phase growth in H^3-thymine for many generations and transferred to $+H^3T-AU$ medium. Subsequently, autoradiographs were made of microcolonies derived from such cells. The dispersion of label during growth to microcolonies permitted higher grain counts and considerably greater resolution of the homogeneity or heterogeneity of the content of label in the population than scoring over single cells as done by Hanawalt, Maaløe, Cummings, and Schaechter (12).

Fig. 2 shows grain count distributions for colonies grown from fully labeled

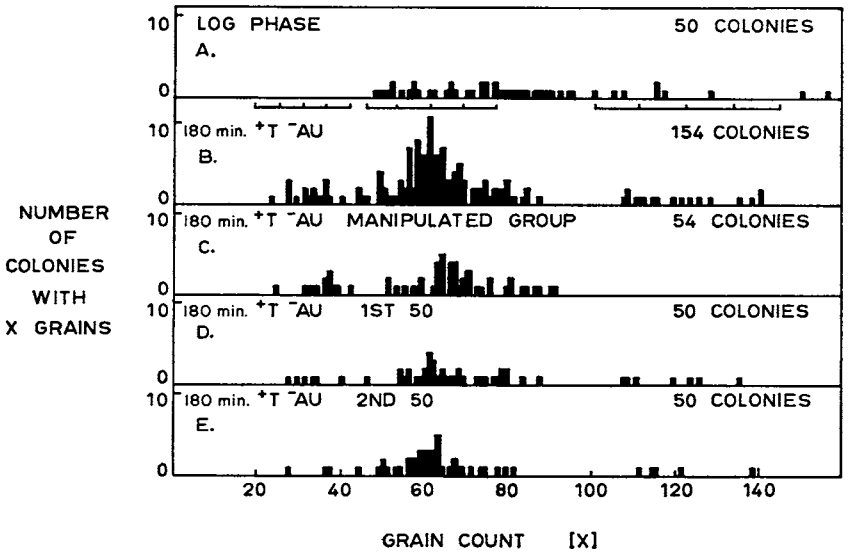


FIGURE 2 *E. coli* $15T^-A^-U^-$ was grown overnight at 37°C in C-glucose medium in the presence of arginine, uracil, and H^3-thymine from a titer of 10^8/ml to a titer of 10^7/ml at a specific activity of 1.7 C/mmole. After washing, the cells were further incubated in medium with H^3-thymine of the same specific activity but lacking arginine and uracil for 180 minutes. Washed cells were then grown on nutrient agar for subsequent autoradiography of colonies (*B, D, E*) and micromainipulated progeny lines (*C*). The autoradiograph films were exposed for 18 days. In (*B*), the expected 1σ and 2σ error ranges are indicated for a hypothetical population of classes with means in the ratio 1:2:4 with the average for class 2 taken as 61 grains. For (*A*), *E. coli* $15T^-A^-U^-$ was grown overnight as above except that the specific activity was 2.9 c/mmole and the titer was 10^8/ml. After washing, cells were grown on nutrient agar for colony autoradiography. Autoradiograph exposure was 16 days.

+T−AU cells in comparison to that from a fully labeled log-phase population. As one might expect, for a log-phase population with cells continuously synthesizing DNA, the distribution of DNA content is broad with no obvious class structure (*A*). For +T−AU cells (*B*), however, the situation is different. The distribution appears to be both more bunched and peaked. A large fraction of the population falls near a peak at about 61 grains. If a class of cells with a homogeneous content of label produced this peak, one would expect that 95 per cent of the cells in that class would have grain counts within ± two standard deviations of the peak value (*i.e.*, $\pm 2(61)^{1/2}$). About two-thirds of the total population fall within these limits. A clearly separate class of cells falls within similar limits about a value twice the peak value of the largest class. Finally, most of the remainder are clustered near a value one-half that of the main class. Roughly then, the distribution is consistent with decomposition into three classes with grain counts in the ratio 1:2:4. One might expect these classes to be homogeneous populations if the DNA of the cells resides in integral numbers of completed discretely sized chromosomes. One cannot argue with the precision concerning this point from these data alone but the tailing of the main peak toward higher grain count values speaks against perfect homogeneity and thus against perfection of chromosome completion. About three times as many colonies were scored for +T−AU than for the log-phase distribution shown. Distributions (*D*) and (*E*) are the first and second groups of fifty +T−AU colonies scored indicating that one can see the differences from the log-phase case even with fewer total colonies.

Examination of the mode of segregation of label in progeny lines isolated by micromanipulation further distinguished the classes suggested by total grain count alone. Small to medium sized, fully labeled cells previously incubated for 180 minutes in +T−AU were selected for initiation of progeny clones. Progeny isolations were performed for four to five generations and terminal isolates permitted to form small microcolonies (4 to 8 cells) before stopping growth. Grain counting of subsequent autoradiographs permitted reconstruction of the distribution of label to progeny. The progeny assignments of grains were made not only for the generations for which isolation was performed but also for the siblings in small terminal colonies. Such scoring of terminal colonies is possible because the orderly arrangement of cells permits reconstruction of sibling relationships and the range of tritium electrons is short.

The total grain count distribution of these manipulated clones is shown in Fig. 2*C*. Since cells for manipulation were selected from approximately the smaller half of the length distribution of the population, it is not too surprising to find no examples of the 4*n* class.[1] The other two classes are present. Their average grain counts appear slightly higher than for the non-manipulated case, possibly because

[1] The letter *n* is introduced here for simplicity of exposition by analogy with its use to denote the haploid chromosome set in other biological organisms.

of somewhat greater efficiency in both registry and scoring for the smaller terminal colonies resulting from micromanipulation. For the most part, 1*n* clones arose from the shortest cells isolated and 2*n* clones from somewhat longer ones. Fig. 3 shows label segregation patterns for the first few generations for some clones of the two major classes. These particular clones were selected to illustrate that it is relatively easy to find clones of each class whose segregation patterns are those expected of cells containing 1 and 2 fully labeled bipartite chromosomes. 1*n* clones generally distribute label approximately equally at the first division and often very unequally at the second generation. Excepting single grains from consideration, (see caption to Fig. 3) these unequal segregations are often all-nothing. For 2*n* clones, unequal segregations occur first at the third generation. It should be emphasized that unequal segregation of label does not occur for all cells of all clones at the generations noted above. Superposition of the fragmentation process, mentioned previously, reduces the frequency of unequal segregations.

The results are consistent with the view that conditions can be found in which fully labeled cells behave as if they contained just two segregative subunits. However, Fig. 4 illustrates an alternative interpretation of second generation all-nothing segregation based on the notion that a cell with 1*n* amount of DNA contains more than one duplex unit (2 in the figure). Without fragmentation, the presence or absence of equal segregation of label at the second generation would distinguish the model of Fig. 4 from the single duplex model. In the presence of fragmentation a more elaborate statistical evaluation becomes necessary (Table I). The analysis indicates that cells with the 1*n* quantity of label too often distribute this label unequally to second and third generation daughters to be consistent with the model of Fig. 4. This conclusion holds whether or not single grains are considered indicative of chromosomal label. Models invoking a larger number of units than the 2 of Fig. 4 clearly are also incompatible. A variation of Fig. 4 involves two hypothetical duplexes which are not of equal size. A relatively high frequency for successive all-nothing segregations of label does not favor such a model and calculations not detailed here set an upper limit of 30 per cent of the 1*n* quantity of DNA for the size of the smaller of two such units.

As with non-manipulated colonies, the 2*n* manipulated class tails to higher grain counts than would be expected for a homogeneous population. The actual values in both cases extend close to mean values expected for cells containing three chromosomes. Several cases in the micromanipulated group with total counts close to 3*n* had segregation patterns suggesting this interpretation. In two of these cases, the first division of the isolated cell on agar produced daughters of unequal length, one approximately twice as long as the other. The longer daughters then proceeded to produce a subline whose divisions were approximately one generation advanced in time over the shorter cell subline. The segregation patterns of these two clones are shown in Fig. 5. In each case the larger first daughter received the most label.

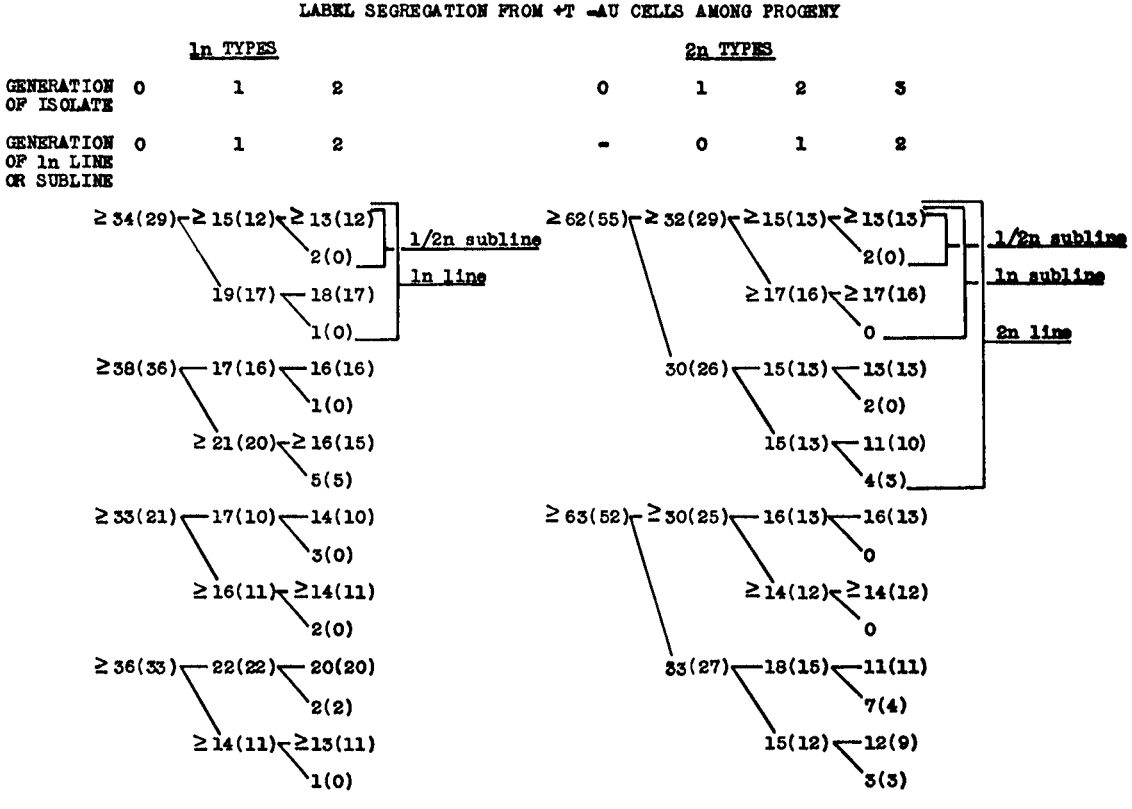


FIGURE 3. Definitions of $1n$ and $1/2n$ lines and sublines and the generation designations thereof are indicated in the figure.

Grains over terminal microcolonies appear singly or in clusters of two or more. Grains lying more than about one micron from any other were scored as single grains. Some single grains are background and uncertainty exists whether all other single grains arise from chromosomal label (see text consideration of fragmentation). Numbers listed in parentheses are values obtained if all single grains scored at sixth generation of $1n$ lines or sublines are omitted.

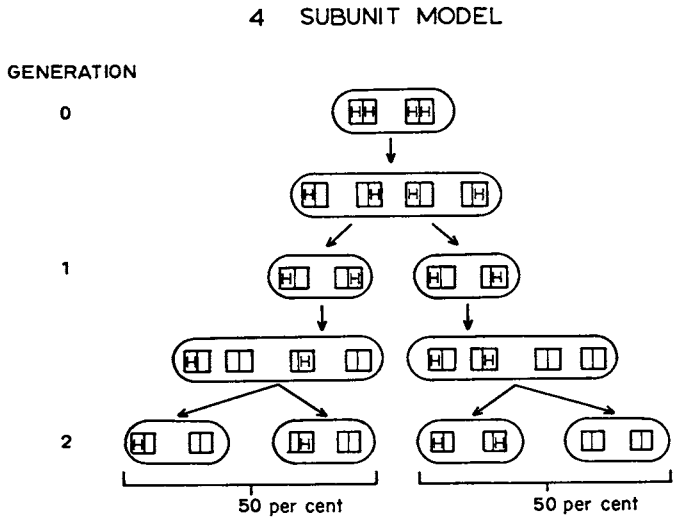


FIGURE 4 Model depicting the segregation of two labeled (H) duplex chromosomes in which two of four progeny chromosomes are distributed to daughter cells at random.

Though the division of label appears to be somewhat greater than 2:1 in each case, the observation is still statistically indistinguishable from this ratio ($\chi^2 = 2.5$, $p = > 0.25$ for two degrees of freedom). In each case, the larger daughter first yielded asymmetric segregation of label at its third subsequent division and, in one case, the shorter daughter gave all-nothing distributions in both of its subsequent second divisions.

The finding of clones interpretable as $3n$ had not been totally unexpected. In experiments in which label was present only during +T−AU incubation or during a second round of +T−AU incubation following a short interval of incubation in +T+AU, autoradiography of single cells (*i.e.*, not grown to colonies) had revealed a few cells where the grains over the cells appeared to be localized at one end of the cell. Autoradiographs of fully labeled log-phase single cells failed to reveal similar cases, arguing against simple displacement of the DNA within cytologically-fixed cells as an explanation of the +T−AU observation. It seems possible that at least some of such cases represent cells which were approximately $2n$ on entry into +T−AU but only one of the two chromosomes proceeded with replication during such incubation. Such an explanation also serves for the $3n$ cases seen with fully labeled cells.

The occurrence of some $3n$ types does not necessarily account for the clones which seem to be intermediate in label between $2n$ and $3n$. Although Fig. 1 shows that most of the DNA synthesis in a +T−AU incubation is finished by 120 minutes, if not before, a more sensitive method of detecting residual synthesis was tried. Following +T−AU incubation without label for varying periods of time, aliquots

TABLE I

TEST OF 4 SUBUNIT MODEL

See caption in Fig. 3 for definitions of lines, sublines, and generations.

For each $1n$ line or subline, the distribution of grains between each pair of second generation daughters was indexed as equally or unequally divided. The distribution was considered unequal if the probability of its origin from a process in whch the total grains of the pair are randomly distributed between the daughters is < 5 per cent. The binomial distribution with a chance of success of 0.5 was used to assess this probability. Such random distribution of grains between daughters is expected if the daughters contain equal amounts of radioactivity as would be the case for one-half the second generation daughter-pairs in Fig. 4. Since misclassification of equal cases as unequal would be expected 5 per cent of the time on the above basis, a correction of the final number considered unequal was made by transferring 5 per cent of this number to the equal class. A χ^2-calculation was then made on the resulting numbers of unequal and equal splits for the chance that these would be drawn from a population with equal numbers of the two types of splits (prediction of model in Fig. 4).

Because the origin of single grains in progeny-line distributions is not certain (see text consideration of fragmentation), their inclusion might bias against recognition of unequal segregations by the above procedures. Their exclusion should not bias the detection of equal segregations since single grains occur with approximately the same frequency in sublines with and without other label. The calculations were thus made both for the case where all grains were tabulated and the case where single grains at the 6th generation of $1n$ lines or sublines were omitted.

According to the model of Fig. 4, label apparently conserved in any given generation after the first should be shared between daughters at the next subsequent generation 50 per cent of the time. For the third column of the table, second generation daughter-pairs unequal at the 1 per cent level by the binomial test were identified. The nearly conserved daughters of such a pair were examined as above for the equality of label distribution to 3rd generation daughters. All grains were used in this tabulation.

	2nd generation all clones All grains		≥2 grains		3rd generation from 2nd generation unequal cases	
Number $1/2n$ clones examined	139		139		53	
	Observed	*Corrected*	*Observed*	*Corrected*	*Observed*	*Corrected*
Number unequal. Binomial test at <5 per cent	85	81	89	85	44	42
Number equal	54	58	50	54	9	11
χ^2 for 50:50 corrected data	3.8		6.9		18.2	
Probability—χ^2	0.05		<0.01		≪0.01	

of cells were incubated for an additional 180 minutes with H^3-thymine in the absence of arginine and uracil. Autoradiographs of colonies from cells placed on label as late as 210 minutes after beginning +T−AU incubation revealed measurable quantities of clustered label in colonies from some cells. Likewise, autoradiographs of single cells not grown to colonies revealed some labeled cells among many unlabeled cells. The rate of such residual synthesis in general is slower

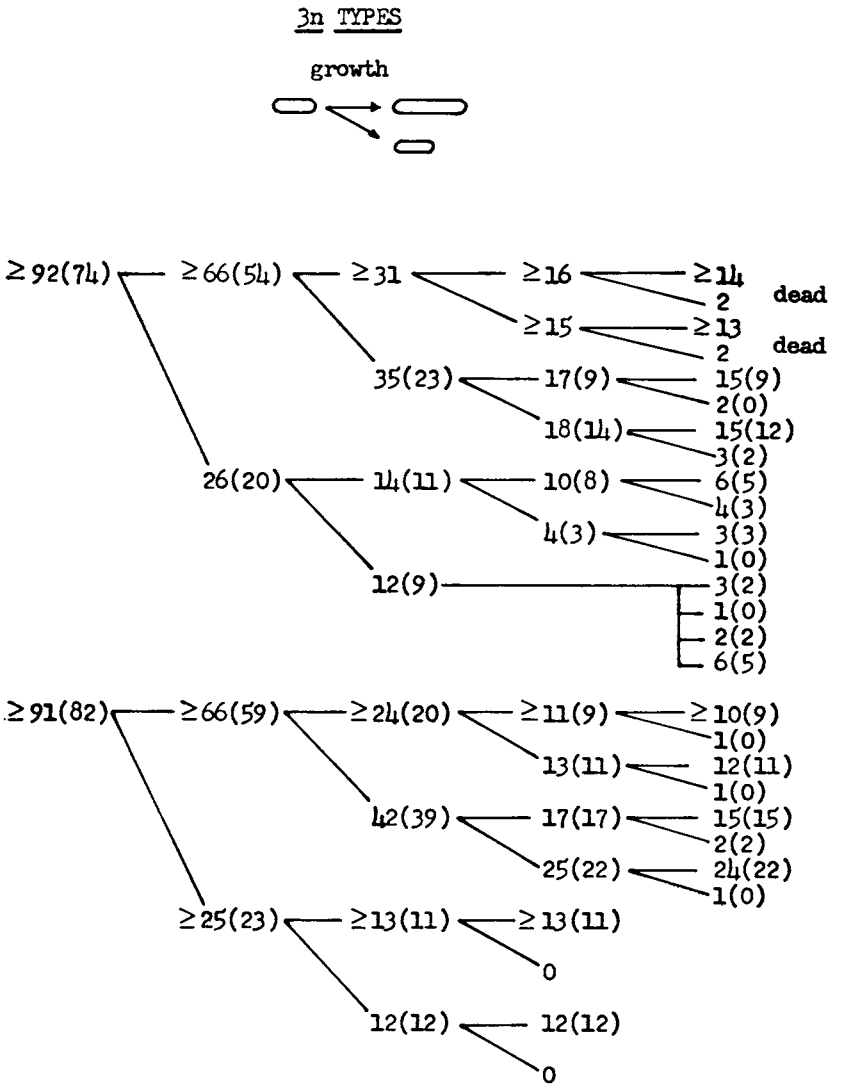


FIGURE 5 See caption to Fig. 3 for explanation of numbers.

than log-phase or early +T−AU synthesis since labeled colonies usually contained much less label than the 1*n* amount. In a 180 minute period of exposure to label late in +T−AU incubation, it is estimated that roughly 10 to 25 per cent of the cells have undergone some DNA synthesis. This failure in perfection of blocking DNA synthesis accounts at least in part for the clones intermediate between 2*n* and 3*n* in the fully labeled +T−AU experiments. Experiments with *E. coli* 15T⁻H⁻ indicate behavior similar to *E. coli* 15T⁻A⁻U⁻ in that during +T−H incubation

the rate of DNA synthesis as measured by H^3-thymine incorporation shows kinetics similar to Fig. 1, but autoradiography again indicates the block in synthesis is not complete in all cells.

It is worth noting that fully labeled cells of *E. coli* 15T$^-$ segregating label primarily in a semiconservative fashion have been found under growth conditions (phosphorous limitation, early resting phase) in which the average cell size of the population is reduced compared to log-phase cells. Under such conditions and for fully labeled cells subject to amino acid starvation, search has failed to reveal clones in which only one daughter receives all or most of the label. Absence of such "one-spot" clones under fully labeled conditions which do yield clones with two labeled cells fortifies the idea that the smallest packaging of DNA for a viable cell is one duplex chromosome.

Characterization of Fragmentation. The studies reported above corroborate the model proposed by Maaløe and Hanawalt that chromosomes are brought, for the most part, to a state of completed synthesis under conditions of amino acid deprivation. One reason for desiring cells with completed chromosomes has been to permit characterization of chromosome fragmentation as to frequency and the size distribution of the chromosomal segments produced. Though the system is not perfect, the improvement over the log-phase case makes presentation of the results on fragmentation seem worthwhile.

The data are derived from the micromanipulation experiments referred to above in discussion of Figs. 2*C* and 3. Using the terminology outlined in Fig. 3, all lines or sublines initiated from cells with one chromosome were identified by their total grain count (average of 33 grains, range from 22 to 46 grains). Such lines or sublines whose progeny could be scored for 6 consecutive generations were accepted for compilation. This restriction excluded a few lines or sublines which either grew too slowly or had cell death and/or filament formation in their lineage. One clone was discarded because of confusion in progeny line cataloguing. Another clone was omitted because the amount and segregation of label did not follow predominant patterns described above. Fig. 6 shows the pattern in this clone and serves to illustrate that there exist occasional segregation patterns which are not compatible with the simple models which accommodate most of the data. Whereas the grain count of this clone is in the range of the $2n$ class, unequal segregation of label occurred at the second generation.

Fig. 7 shows the distribution of grains among sixth generation daughters from the 69 $1n$ lines and sublines suitable for compilation. This distribution is of the general form expected for fragments produced by random breakage of linear polymers—that is, the frequency of fragments is inversely related to their size. For comparison with the data, theoretical plots of distributions of grain counts resulting from breakage of uniformly labeled homogeneous linear polymers are plotted in the figure. These were calculated from the theoretical expressions of

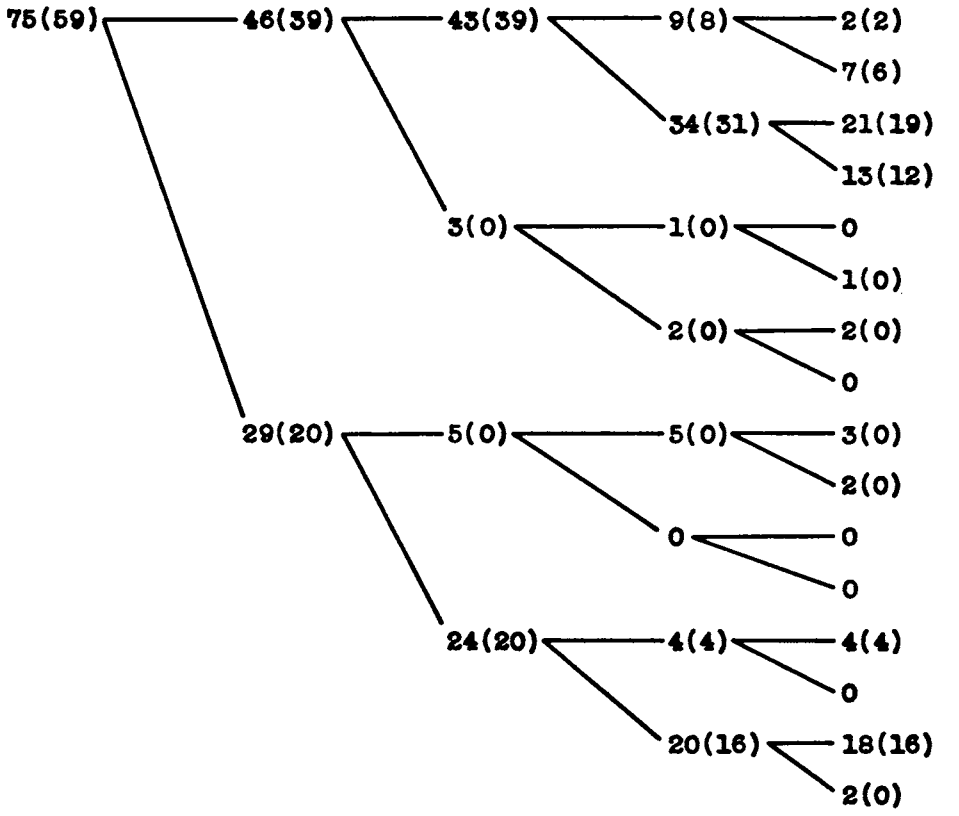
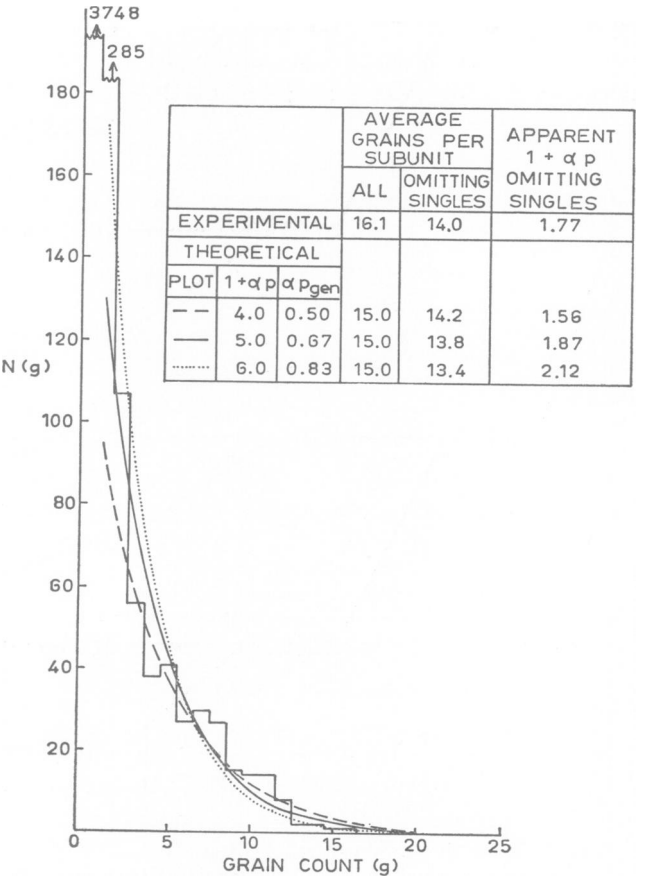


FIGURE 6 Unusual clonal segregation of label. Data shown through generations for which manipulation was performed. See Fig. 3 for explanation of numbers.

Montroll and Simha (18) using values of breakage consistent with the frequency of fragmentation estimated from our data. The notion in making such a comparison stems from the conjecture that though fragmentation occurs in successive generations, if the process involves single random interruptions in a linear structure (or a circular structure with a point of closure which opens once each replication), then the particular generation of origin of an interruption within the original labeled chromosomal material will be irrelevant to the final distribution of label. The assumption is made, however, that all interruptions yield fragments which segregate into separate daughter cells. This is certainly suspect because of the possibility of multiple events (if, for example, sister-strand crossovers) leading to reunion of otherwise separable fragments. Nevertheless, the data fall close to the expectations of a random break model. Single grains, which account for 12 per cent of the data, seem to be too frequent to be consistent with the model, however. The theoretical curves shown are based on the arbitrary omission of 50 per cent of the



			AVERAGE GRAINS PER SUBUNIT		APPARENT 1 + α p
			ALL	OMITTING SINGLES	OMITTING SINGLES
EXPERIMENTAL			16.1	14.0	1.77
THEORETICAL					
PLOT	1 + α p	α p_{gen}			
– –	4.0	0.50	15.0	14.2	1.56
——	5.0	0.67	15.0	13.8	1.87
·····	6.0	0.83	15.0	13.4	2.12

FIGURE 7 Frequency distribution of labeled cells for 69 clones after 6 generations of growth. Bar-graph depicts experimental results corrected for background. Background was estimated by counting grains over measured areas of agar adjacent to scored colonies, estimating the area of all colonies scored and subtracting the calculated contribution from the category of cells containing only one grain. Also shown are theoretical plots of the frequency distribution of grain counts, $N(g)$, to be expected from the random breakage of labeled and initially homogeneous polymer molecules. These were calculated, using a 7094 computer, from the expression

$$N(g) = \sum_t N(t) \cdot P(g)_{t \cdot g_{t-1}},$$

where $N(t)$ = frequency distribution of polymers, t monomers long, resulting from the breakage

and $P(g)_{t \cdot g_{t-1}}$ = Poisson distribution of grains, g, for average number of grains, $t \cdot g_{t-1}$, where g_{t-1} is the average grain-count per monomer, $t = 1$.

Montroll and Simha (18) have shown that

$$N(t) = \alpha(1 - \alpha)^{t-1}[2 + (p - t)\alpha] \qquad t \leq p$$

$$N(t_0) = (1 - \alpha)^p \qquad t_0 = p + 1$$

single grain activity. The table in Fig. 7 indicates that a fragmentation frequency of 0.5 to 0.7 per generation gives predictions reasonably close to the data for the grain count and number of fragments per chromosomal subunit exclusive of single grains. Theoretical curves based on all single grain activity do not give as good agreement.

Several factors might contribute to single grain bias. These include technical items such as the method of scoring grains, the presence of non-DNA label, and the heterogeneity in the degree of labeling of subunits arising from incomplete blocking of DNA synthesis. Biological possibilities are the presence of non-chromosomal DNA, and a replication or exchange mechanism favoring the production of small fragments. The precise role of each is difficult to assess.

Examination of the grain count distribution among cells of the generation representing the termination of micromanipulative isolation, indicates less bias toward single grains. This suggests that scoring over terminal clones leads to an overestimation of the number of single grains arising from separate loci of radioactivity.

Concerning the identification of label as DNA, exposure of coverslips bearing single cells or microcolonies grown from cells labeled with H^3-thymine to DNAse leaves an autoradiographic residual activity of 2 to 4 per cent compared to buffer treated controls. This is consistent with the view that a considerable fraction of the low grain count activity is from DNA at the time of autoradiography—*i.e.*, after growth to microcolonies.

Some effort has been made to examine the possibility that *acid soluble* H^3-material present in the cells when initially isolated from radioactive medium becomes incorporated into DNA during growth on non-radioactive agar. Evidence for such transfer was obtained when cells incubated with +T−AU for 180 minutes without label were then continued 180 minutes more in the presence of H^3-thymine. After harvest, labeled cells were deposited on non-radioactive blocks on glass slides cut to a size suitable for insertion in a gas flow Geiger counter. Growth of the cells was stopped at various times and the glass planchets fixed as for autoradiography (which removes acid soluble material) but instead their activity was measured by Geiger counting. Table II shows that there was an increase in the amount of label retained in the cells as they grew. Similar results have been obtained by autoradiography where the average grain count per day for single cells was less than for colonies. Estimated from this difference in grain count, the extent of such

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where p = number of bonds in original polymer and α = probability a given bond will be broken so that αp = average number of breaks per original molecule and $1 + \alpha p$ = average number of fragments of all sizes per original molecule. For the computations, t_0 was set = 201. This value, of course, is very much less than the number of nucleotide pairs in the DNA of the chromosome. Such a number would require excessive time for computation. Examination of the dependence of $N(g)$ on t_0 indicated that the value chosen was a suitable approximation to t_0 very much larger. The average grain count of the initial polymer was taken to be 15.0, giving a monomer grain count $g_{t-1} = 15.0/201 = 0.075$.

TABLE II

TRANSFER OF ACID-SOLUBLE LABEL

Each value represents the average of counts from five planchets.

Time of growth	Average
minutes	CPM
0	151
30	154
60	166
120	213
240	237
360	246

TABLE III

EXAMINATION FOR CORRELATION OF FRAGMENTATION IN SUCCESSIVE GENERATIONS

For terminology see caption to Fig. 3.

It was desirable to minimize the scoring of sublines containing DNA which might have arisen from imperfect blocking of DNA synthesis since segregation of such DNA might be misconstrued as fragmentation. To this end, $1/2n$ sublines were selected in which the chromosomal subunit appeared conserved at the second generation of the $1n$ lines or sublines of which they were a part. Fragmentation in pairs of consecutive generations were tabulated as present (+) and absent (—) for all pairs in generations 3 through 6 in which no preceding fragmentation had occurred in the pair's subline. In (*A*) single grains appearing at the 6th generation of $1n$ lines or sublines were omitted. For (*B*) only single grains arising at the second generation of $1n$ lines or sublines were omitted. Without this exclusion, $1n$ lines or sublines segregating a completely unlabeled daughter at the second generation were too few for analysis. Such selection does permit examination of the effect on the results of indexing segregation of single grains as fragmentation.

The numbers of each type of pair are compared with the numbers expected from a binomial distribution with a probability of success equal to the average fragmentation frequency of the data.

	A		B	
Fragmentation in pairs of consecutive generations	Actual number of pairs	Expected number of pairs	Actual number of pairs	Expected number of pairs
+, —	25	22	12	14
—, +	24	22	12	14
+, +	7	9	18	16
—, —	51	54	13	11
Average fragmentation frequency per generation	0.29		0.55	

transfer is small relative to the total nucleic acid of a cell and would account for less than 5 per cent of the label shown in Fig. 7. The transfer which takes place slowly over several generations would be expected to label subunits synthesized during this time at low specific activity and thus contribute to the frequency of single autoradiograph grains.

From the above observations plus an expected contribution from incomplete blockage of DNA synthesis, there appear to be substantial sources of excess single grain activity without involving potentially more interesting biological possibilities.

It was of interest to examine the progeny line data from the fully labeled +T−AU experiments already discussed for possible correlation of dispersive replications in successive generations. From the data presented in Table III, no such correlation is demonstrable whether or not single grains are included in the analysis.

Table IV shows the results of an experiment designed to explore the effect of variation in specific activity of the H^3-thymine used for labeling on the fragmentation frequency. Use of the fully labeled +T−AU system permitted classification of clones by chromosome content, thus permitting evaluation of the fragmentation frequency per chromosomal subunit. The ratio of specific activity achieved was judged from the relative authoradiograph times needed to bring the distributions to the same average grain count per chromosome. The results indicate about a 20

TABLE IV

FRAGMENTATION AT DIFFERENT SPECIFIC ACTIVITIES

Cells fully-labeled in log-phase were further incubated 180′ in +H^3T-AU. After washing samples of each culture were grown to microcolonies for the same period of time and subsequently autoradiographed. The actual specific activity ratio achieved, as judged by grain density, was less than planned. The areas of colonies were estimated with a Whipple reticle in an eyepiece of the microscope and give an indication that scoring was performed for clones of similar average growth. Roughly, two Whipple units correspond to five generations of cell growth. The number of fragmentations per subunit was taken as equal to N_L-2P/2P, where N_L = number of labeled locales and $P = 2$ or 4 for the $2n$ and $4n$ types of clone respectively.

Intended specific activity C/mmole	3.3		0.6	
Exposure—days	6		25	
Average grain count $2n$	41.2 (67 clones)		38.6 (39 clones)	
$4n$	83.6 (25 clones)		75.6 (8 clones)	
Actual specific activity ratio	4.4		1	
	$2n$	$4n$	$2n$	$4n$
Average clone area (Whipple area)	2.3	3.4	1.9	3.8
Average number labeled locales				
All grains	14.2	26.7	12.7	25.0
Excluding singles	9.2	18.2	8.1	16.3
Average number of fragmentations per subunit				
All grains	2.5	2.3	2.2	2.1
Excluding singles	1.3	1.3	1.0	1.0

per cent lower frequency of fragmentation with a 4.4-fold reduction in specific activity. Barring an unusual dose-response effect for fragmentation, this argues that most of the dispersion arises from processes not related to the tritium decays occurring during the experiment.

DISCUSSION

That a large duplex structure whose primary form of replication is semiconservative is the fundamental organization of DNA in *Escherichia coli* has received support from experiments with fully labeled cells subjected to amino acid starvation and to culture conditions producing shorter cells. The class structure of grain count distributions following +T−AU incubation suggests that this structure may reasonably be considered to have a discrete physical size although the precision of such discreteness is not well defined by the data. Statistical evaluation of label segregation patterns render it unlikely that the quantity of DNA associated with a semiconservative pattern of label distribution (1*n* amount) represents more than one duplex chromosome. These results substantiate previous inferences concerning the organization of bacterial DNA drawn from comparison of label segregation patterns from partially and fully labeled log-phase cells (3).

From the autoradiograph exposure time and the estimates of specific activity used in the +T−AU experiment reported, one can calculate a grain count of 1.0 grains per C/mmole per day of exposure for the chromosome from the non-manipulated clones of Fig. 2 and a value slightly higher for manipulated clones. This is in reasonable agreement with the autoradiographic size of the chromosome deduced from manipulation-segregation studies on strains of *E. coli* K12 (8). No independent estimate of the DNA content of *E. coli* cells has been made in connection with these experiments. Reference can be made to values reported by others (16, 19, 20) for log-phase cells in the same or similar medium as used here. Using these values and comparing the grain count per chromosome from a +T−AU culture and the grain count per clone of a log-phase culture from data such as those in Fig. 2 (each normalized to grains per unit specific activity per day of exposure), the duplex unit can be estimated to have a size of $2.2 \pm 0.7 \times 10^9$ daltons (for a log-phase culture range of $5\text{-}9 \times 10^{-15}$ gm DNA per cell). This would be a Watson-Crick B-form length of $1200 \pm 380\mu$ which can be compared with the value of 1100μ reported by Cairns (7) for the length of the non-replicating form of isolated *E. coli* chromosomes. This agreement and the applicability of the results reported here to the cell population as a whole support the view that the chromosomes observed by Cairns are typical of the population and that the units conserved in replication are the individual polynucleotide chains of the duplex DNA molecule.

The method of counting grains over microcolonies derived from single cells permits the scoring of many more grains than possible by counting the cells without such growth. This has made possible the observation of class-structure heterogeneity

in a thymine-saturated, amino acid-starved population. Whether such class structure would apply to cell populations under slightly different experimental conditions such as used by Maaløe and Hanawalt is not clear. Our results, particularly the occurrence of the 1*n* and 3*n* cells, do suggest, however, that cell division is not a necessary or sufficient condition for the initiation of a round of chromosomal replication. Neither must cell division await the start of new rounds of DNA synthesis in both daughter chromosomes. These conclusions are consistent within the well established facts of variation in size, DNA content and nuclear body content shown under various environmental conditions (*e.g.*, 21). Thus, the conjecture that regulation of cell division and chromosomal replication in *E. coli* is a mutually dependent phenomenon (22) is doubtful as a generalization. This is not to argue against the liklihood of a relationship between the chromosome and some surface element possibly also involved in surface growth (23).

The fact that 3*n* clones occur following +T−AU incubation, may have particular bearing on the problem of control of chromosome replication. Of course, such cells might have arisen from 4*n* cells which divided asymmetrically, 1*n* and 3*n*. However, the observation of label clustered over one end of occasional single cells incubated with label only during amino acid-deficient conditions, suggests a different interpretation. This is that one of the two daughter chromosomes of a cell initially close to 2*n* at the start of +T−AU proceeds with the next round of replication while the other chromosome remains blocked by the amino acid-deficient state. This nonsynchronous initiation of replication may have occurred either prior to imposition of the amino acid deficiency or may represent independent escape from the block. In either case, such independence of initiation and continuation of synthesis of chromosomes argues that replication of chromosomes lying in the same cytoplasm is, or can be, individually controlled. This control might result from some feature of the chromosome itself. On the other hand, if a nonchromosomal constituent interacting with the chromosome is responsible for the control, the observation suggests the presence at most of a small number of such molecules per cell under conditions of amino acid starvation. Recently Pritchard and Lark have presented evidence that new rounds of synthesis are initiated by thymine deprivation in T⁻ strains (14). Although our cells were grown in 2 γ/ml of thymine, it is possible that this concentration of thymine would still permit an occasional initiation event resulting in 3*n* cells.

The imperfection of the +T−U state in completely stopping DNA synthesis was a disappointment for certain projected experiments. Whether such synthesis represents residual synthesis of chromosomes initiated prior to amino acid deprivation or initiation of new synthesis by escape from the imposed block is not yet certain. Possible leakiness in the mutants [*E. coli* 15 TAU is known to be leaky (24)], or protein turnover (25, 26) might contribute to such escape. Increasing thymine concentration to 20 γ/ml did not prevent residual synthesis, making it

unlikely new rounds of synthesis initiated by slight thymine deficiency (14) accounts for the observation.

The fragmentation accompanying DNA duplication seems to occur randomly in successive replications and gives a distribution of label among progeny which is not very different from the distribution expected for the random breakage of a linear structure. The origin of this process appears only in a minor way to be associated with tritium decays occurring during the experiments. With the possible exception of the smallest fragments the value of 0.5 to 0.7 fragmenting events per chromosome per generation fits the data reasonably well. Inability to be certain whether bias toward small fragments represented by single grain activity should be considered a feature of the dispersion of chromosomal label leaves open the possibilities that the process is not completely random and occurs with greater frequency. While several experiments in which culture conditions (temperature and growth media) were varied did not produce any easily detectable changes in the frequency, the results of van Tubergen and Setlow (4) indicate a considerably lower value than found here. The reason for this difference has not been discovered.

Infrequent patterns, such as in Fig. 6 are so deviant from the common patterns that they could not be considered to fall within the range of technical error arising in the autoradiography. The scarcity of such clones and the lack of similarity in pattern of segregation in the few deviant clones catalogued from the various experiments performed in this type of work prohibit a unique interpretation of the origin of such distributions. However, puzzling over the patterns observed serves to challenge prejudices concerning the nature of bacterial cells and mode of replication of chromosomes. One wonders about the regularity and equality of segregation of completed chromosomes at cell division, the invarability of the semiconservative mode of replication compared with a conservative process, how clearly separated in time one round of replication is from another (27), and the degree to which the presumably complimentary parts of chromosomal subunits replicate contemporaneously. Although some unusual segregations have been associated with abnormalities (cell death or filamentation) during progeny growth, the cell morphology and timing of cell divisions in the clone of Fig. 5 were quite regular. The pattern of this clone might represent primarily conservative replications with the superimposition of the process causing fragmentation. Alternatively, crossing over between circular daughter chromosomes analogous to the crossing over invoked to explain the kinetics of marker transfer with F′ strains of *E. coli* K12 (28, 29) might result in a chromosome double its usual length. Such a chromosome replicating semiconservatively with fragmentation could yield the pattern observed. Deviant patterns compatible with viability emphasize the plasticity of living processes and the ability of a biological system to adapt and probably often exploit alternative possibilities of molecular behavior even in such a central process as replication of its genetic material.

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