QUANTITATIVE RADIOAUTOGRAPHIC STUDIES ON EXPONENTIALLY GROWING CULTURES OF *ESCHERICHIA COLI*

THE DISTRIBUTION OF PARENTAL DNA, RNA, PROTEIN, AND CELL WALL AMONG PROGENY CELLS

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ABSTRACT Exponentially growing cultures of *E. coli* were examined by quantitative radioautographic techniques to determine the distribution of labeled DNA, RNA, protein, and cell wall among the progeny cells of successive generations. It was found that DNA is in large structures, non-randomly distributed in the progeny. About one-half of the cells have four such structures and approximately one-half contain these four structures plus four smaller ones. These structures show remarkable stability. Fewer than 3.5 per cent of the large structures break in one division time. Protein, RNA, and cell wall are all distributed randomly among progeny cells. The number of units of each component that show random segregation must be 200 or more.

INTRODUCTION

The techniques of tritium radioautography have been successfully applied to the examination of various aspects of the inheritance of labeled parental DNA[1] in *E. coli* (1, 2). We have extended these approaches by using the fact that, under controlled conditions of growth, bacteria divide frequently and uniformly (3). Various cell fractions of *E. coli* were labeled with tritium-containing metabolites. Quantitative radioautographic methods were then used to follow the distribution of labeled DNA, RNA, protein, and cell wall among progeny cells. These experiments give new information about the macromolecular organization in *E. coli.*

Particular cell fractions of selected auxotrophic strains (4, 5) were labeled by

[1] The following abbreviations are used: deoxyribose nucleic acid (DNA), ribose nucleic acid (RNA), α,ϵ-diaminopimelic acid (DAP), tritiated thymidine (H^3T), tritiated uridine (H^3U), division time (τ).

use of purified radiochemicals which satisfied the growth requirements of the bacteria. The bacteria were grown for 10 generations in the presence of excess specific radiochemicals. At this time the supply of radioactive label was cut off and the cells permitted to manufacture macromolecules and divide exponentially. At each subsequent division time, samples of the cell population were removed and used to make radioautographs. Each generation of progeny cells was characterized by observing and tabulating the number of photographic grains associated with single cells. By analyzing the *distribution of grains among the cells* for successive generations, we have arrived at estimates of the average number of macromolecular units making up particular cell fractions in *E. coli.*

We shall be concerned only with cells which were initially fully labeled and shall confine our attention to (*a*) which macromolecular fraction or fractions contain the label, (*b*) whether the label is conserved in the process of cell growth, and (*c*) how the label is distributed among progeny cells growing in the absence of labeled metabolites.

MATERIALS AND METHODS

I. *Bacterial Strains, Media, and Growth Conditions*

The auxotrophic strains used and their specific biochemical requirements are shown in Table I. All cultures were initiated from single-cell clones grown on nutrient agar. They were grown in 1.0 ml volumes, without aeration in 1 cm diameter test tubes, at 23.5°C unless otherwise stated. The biochemical requirements were met by the addition of the radiochemicals, shown in Table II, plus unlabeled lysine (4000 μgm/ml) and arginine (80 μgm/ml) for mutants 173:25:DAP⁻ and 15T⁻U⁻A⁻, respectively. When H^3-proline and H^3-leucine were used, they were at concentrations of 200 μgm/ml and 12 μgm/ml, respectively. Growth in minimal medium at 23.5° was useful for our purposes because

TABLE I

STRAINS OF *E. coli* AND THEIR GROWTH MEDIA

Strain	Reference No.	Requirement	Growth medium*
		μgm/ml	
15T⁻	6	4 thymidine	Minimal
15T⁻U⁻	7	4 thymidine 20 uridine	Minimal
15T⁻U⁻A⁻	8	4 thymidine 20 uridine 80 L-arginine	Minimal
173 : 25 : DAP⁻	9	10 DAP[1] 20 L-lysine	Complete

*Minimal medium: M9 containing 0.4 per cent glucose (10). Complete medium: 0.3 per cent yeast extract, 0.8 per cent nutrient broth, 1 per cent glucose.

TABLE II

DESCRIPTION OF RADIOCHEMICALS USED IN GROWTH MEDIUM

Radiochemical	Method of labeling	Specific activity	Probable *in situ* site of label
		mc/mM	
H^3-α, ϵ-diaminopimelic acid	Exchange in tritium gas*	430	Cell wall
H^3-DL-leucine	H^3-methylation	380	Protein
H^3-L-proline	Exchange in tritium gas*	42	Protein
H^3-thymidine	Catalytic exchange‡	890	DNA
H^3-uridine	Catalytic exchange‡	135	RNA

* Reference 11.
‡ References 12, 13.

it gave exponential growth up to cell concentrations of 10^8/ml, and over 95 per cent of the cells had length between 1.2 and 2.4 μ. Only 1 per cent of the cells were double cells ($\sim$ 4 μ long) and less than 0.1 per cent were filamentous. If cells were grown at 37°C, over 20 per cent of them fell outside the size range given above, and if grown at temperatures below 23°C they yielded cultures which had irregular and varying division times.

The radiochemicals, with the exception of H^3U, were purified by paper chromatography, on Whatman No. 1 paper, using the solvents shown in Table III. All radiochemicals were obtained from the New England Nuclear Corporation, Boston.

TABLE III

THE SOLVENT SYSTEMS USED IN THE PURIFICATION OF RADIOCHEMICALS

Compound	Mode	Solvents	Volume ratios
H^3 T	1 dimension, ascending	Isopropanol/conc. NH_4OH water (14)	70/5/25
H^3-leucine	2 dimensions, ascending	(*a*) Sec. butanol/HCOOH/water	70/10/20
H^3-proline		(*b*) 1 lb. carbolic acid + 115 ml conc. NH_4OH/water (15)	1.6/113
H^3-DAP	1 dimension, descending	Ethanol/water/10 M HCl/pyridine (16)	80/17.5/2.5/10

II. *Experimental Procedure*

Cells were grown to a concentration of 10^8 cells/ml in unlabeled medium and then diluted by a factor of 10^8 into labeled medium. They were further grown to a concentration of 10^8 cells/ml. Such cells will be referred to as "fully labeled" cells. Fully labeled cells were diluted 10 times into unlabeled medium containing 100 times the concentration of unlabeled metabolites, or, in experiments to be carried out beyond 5 division times, the cells were centrifuged free of the labeled medium and resuspended at about 1/100 the original concentration in medium containing a 100-fold excess of unlabeled metabolite. These procedures cut off the supply of labeled metabolite to the growing cells. Cell growth, which continued normally, was measured as a function of time by counting cells in Petroff-Hauser chambers. At successive times after resuspension in unlabeled

medium, cells were removed and processed for radioautography, or counting in a Geiger counter, as described below. The times of removal of samples coincided, whenever possible, with the successive average times of cell division.

III. *Geiger Counting*

Labeled cells were treated in the following way. Known volumes (0.005 to 0.05 ml) of cells were added to 0.05 ml of water placed on stainless steel planchettes. A drop of 0.2 per cent formalin was added and the solution was evaporated to dryness at 50°C and the samples, on the planchettes, then fixed in Carnoy's solution (17) and graded alcohols (18). After drying, the samples were counted in a windowless flow counter through which flowed "Q" gas (Nuclear Chicago Corporation). A Nuclear Chicago Corporation model D-47 tube was used in connection with a Baird Atomic Corporation model 132 scaler. Cells of $15T^{-}$ fully labeled with H^3T gave 7.6 counts/cell/day. Nonspecific adsorption of radioactivity from 0.05 ml of H^3T gave, in all cases, less than 30 counts/min.

IV. *Radioautographic Technique*

Samples were removed with glass loops and placed on subbed glass microscope slides. A drop of 0.2 per cent formalin was added to kill and preserve the samples. The slides were dried and fixed in Carnoy's solution and graded alcohols. After the slides were dried, radioautographs were made with Kodak AR-10 emulsion using the stripping film techniques of Doniach and Pelc (19). The film stripping was carried out at 55 per cent relative humidity in illumination provided by a 25 watt bulb and a Wratten series 1 filter. The film was floated on water kept at 25°C. The majority of the samples were stored for exposure in a dry air atmosphere at 4°C. Most of the seventh and eighth generation samples were stored at 4°C in a CO_2 atmosphere (O_2-free) over $ZnCl_2$ crystals ($\sim$ 15 per cent relative humidity). The latter procedure minimized fading of the latent images (20).

Four different developing procedures at 22°C were used: 8, 4, and 1 minutes in D-19 developer and 2 minutes in D-19b, all followed by ½ minute in dilute acetic acid, 5 minutes in Kodak acid fixer, ½ minute in water, 1.5 minutes in Kodak rapid clearing agent, and 5 minutes in distilled water. Unless otherwise stated a 4 minute development was employed. The autographs were then air-dried, covered with 65 per cent glycerol and a number 1 coverslip. They were examined under oil immersion at 2200 magnification with (unless otherwise noted) a Leitz ortho-lux microscope using Zeiss phase optics. Cells were identified in phase contrast and grain counting carried out with bright field illumination. Background grain density was estimated by counting typical cell-free areas of the emulsion. Eight minute development gave a background of about 1 grain/100 μ^2, 4 minute about 0.5 grain/100 μ^2, 2 minute about 0.2 grain/100 μ^2, and 1 minute about 0.1 grain/100 μ^2. Background corrections to the observed grain counts were calculated from the above data by assuming a sensitive region around each cell of between 2 to 3 μ^2, depending on the number of cells per unit area. In one experiment, using cells grown in the absence of label (Fig. 22), the background was measured by counting grains associated with cells. The observed counts agreed with those calculated from measurements on cell-free regions of the emulsion. The average grain count per cell decreased with decreasing development time. A 1 minute development gave 60 per cent of the average grain count per cell obtained with 8 minute development. The detailed effects of different development and storage conditions will be reported elsewhere (21).

In counting the number of grains associated with a bacterium, the objective lens was brought in and out of focus over the cell under observation. Each resolved silver grain was counted. Those grains which remained as continuous, dark, shifting areas as the focal plane of the objective was raised were scored as single grains regardless of the appearance of the grain pattern in one plane. Otherwise no correction was made for possible tracking in the emulsion. Parts of the emulsions showed latent-image fading and with a 1 minute development time some areas of the emulsion showed labeled cells with extremely small grains and practically no visible background. Only those regions of the emulsions which showed typical backgrounds were used to obtain quantitative data. Little fading was presumed to have taken place if the average cellular grain count decreased by a factor of two per generation. The average for cells fully labeled with H^3T was about 4.5 grains/cell/day with 8 minute development. The response of the emulsion was a linear function of exposure time up to an average grain count of 6 grains/cell.

The data obtained from radioautographs are presented as frequency distribution graphs of the number of cells with a given number of grains per cell.

V. *Enzymatic Treatments*

Solutions of crystalline enzymes (Worthington Biochemical Corporation) in M-9 medium without glucose were pipetted onto warm planchettes or slides containing samples. The samples were incubated at 37°C for known times and the reactions stopped by rinsing the samples in cold water. They were then dried and counted.

RESULTS AND DISCUSSION

I. *General*

We shall describe the distribution of photographic grains among cells in terms of the Poisson distribution function and deviations from this function. This is a useful approach because the random decay process of H^3 is described by this distribution. If, from measurements on a large number of cells, we determine the average number of grains per cell, $\bar{g}$, the probability that a given cell has n grains is given by

$$P(n) = \frac{\bar{g}^{-n} e^{-\bar{g}}}{n!}$$

Such a distribution which, as we shall see, fits the data for fully labeled cells, is a very inflexible one. It is governed only by the value of $\bar{g}$. Moreover, as cells divide in unlabeled medium, the value of $\bar{g}$ should decrease by a factor of two per generation, if no components are lost to the medium.

Now suppose that, as shown in Fig 1*a*, the labeled macromolecular constituents are distributed at random when cells divide. We would expect that the initially observed Poisson distribution, with $\bar{g} = \bar{g}_0$, will, after k divisions, be represented by a Poisson with $\bar{g} = \bar{g}_0/2^k$, if similar exposure and development conditions are used. If, as another limiting case, a cell were to have only two large units which are shared between the two progeny, as in Fig. 1*b*, one would expect to find a Poisson distribution at the time of the first division, 1τ, but not at 2τ. At the latter time only half the cells are labeled and the distribution of grains among the labeled cells

will be Poisson with $\bar{g} = \bar{g}_0/2$. The unlabeled cells have $\bar{g} = 0$. The sum of these two distributions ($\bar{g} = 0$, $\bar{g}_0/2$) is *not* Poisson. We shall therefore say that a sudden appearance in time of markedly non-Poisson distributions implies a non-random distribution of label among progeny, and attempt to use the observed deviations from a Poisson form to calculate the extent of the non-random distribution of labeled macromolecules. If the distribution becomes abruptly non-random at the kth division, there are, on this model, 2^{k-1} units that distribute non-randomly during replication.

There are other possible biological and physical reasons why the observed distribution should deviate from a Poisson. Some cells may not be growing. Radioautographs of 7τ cells used to obtain the distribution of RNA, protein, and cell wall among progeny indicate that a fraction less than 10^{-2} of the cells are not dividing and a double label experiment on cells containing H^3T indicated that over 99 per cent of these cells, up to at least 4τ, were metabolizing and synthesizing

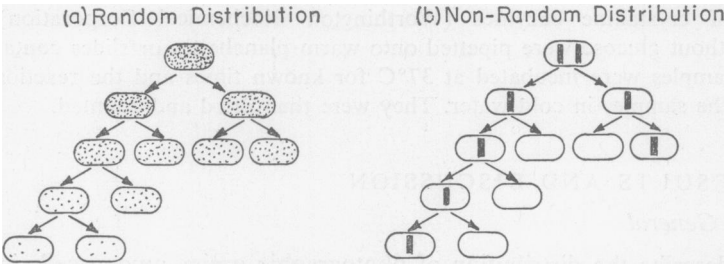

FIGURE 1 Two extreme hypothetical models which show the distribution of parental label among progeny cells. (*a*) A random distribution of many small units and (*b*) a non-random distribution of two units.

RNA and protein. Only at high specific activity labeling for DNA (2.6 c/mM) did we observe the presence of non-dividing cells. Radioautographs of 7τ cells showed approximately 1 in 1000 with 4 times higher activity than other labeled cells. This is probably a radiation effect (22) and we ignored these cells in our grain count data. Another possible reason for the deviations from Poisson is the spread in division times among the bacterial population. Provided that cells do not remember for several divisions what their previous division times were, and experimental evidence from phased cultures (23) indicates that they do not remember, this should have no effect if the proper average division times are used. (Obviously if cells did remember that they were fast dividers, the culture would soon be homogeneous in division times.) Lastly, cells growing in unlabeled medium started with different amounts of label because of the initial size distribution. The general effect of this initial condition is to make the observed distributon at 0τ slightly broader than a theoretical Poisson. The effect of this initial condition on the subsequent distributions of DNA will be taken up below.

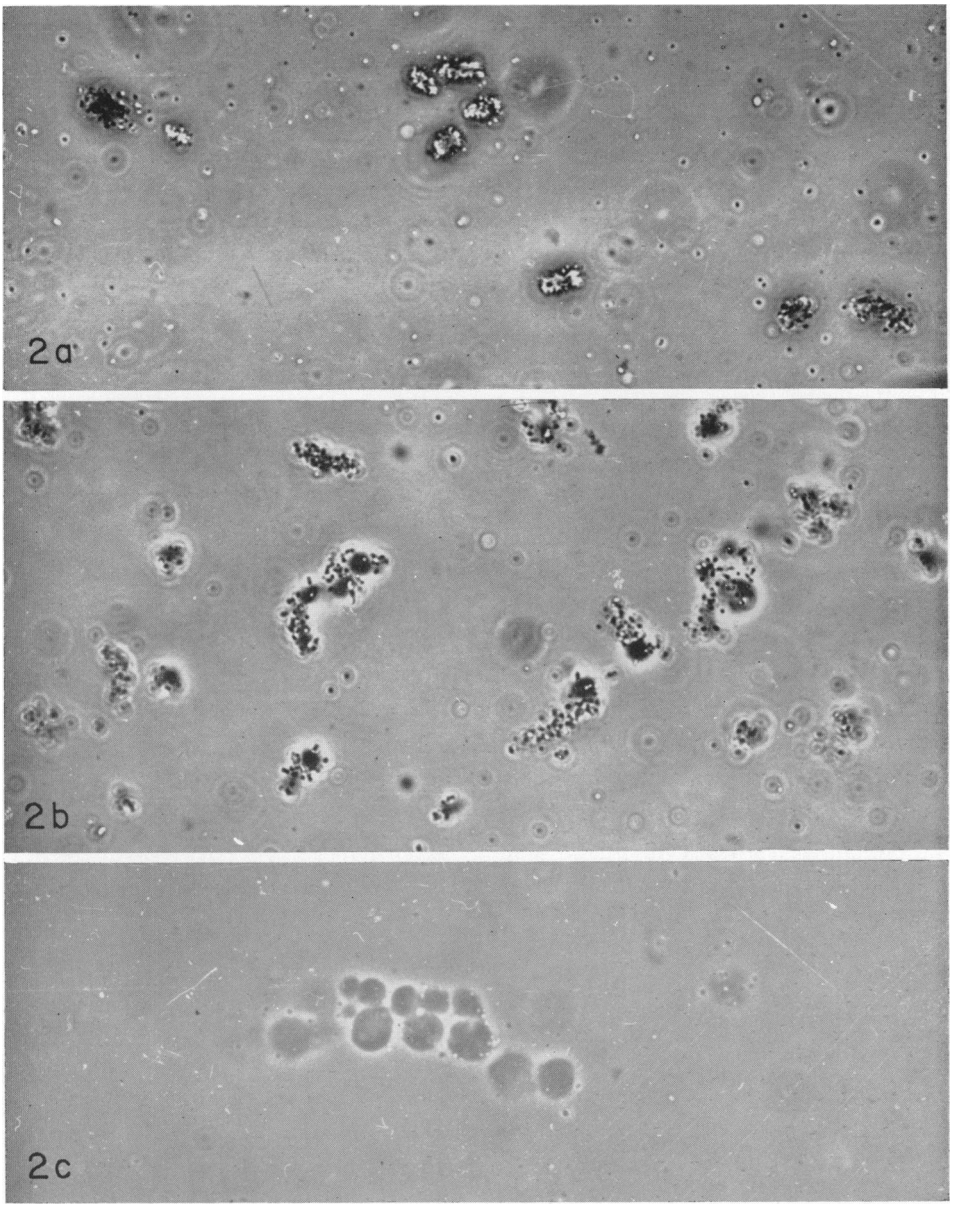

FIGURE 2 Photographs, in phase contrast, of *E. coli* 173:25:DAP$^-$ after labeling with H^{3}DAP. (*a*) fully labeled cells, (*b*) emerging protoplasts 8 hours after DAP is removed, and (*c*) developed protoplasts of the same cells 16 hours after DAP is removed. The grains appear as white spots in 2*a* and 2*c*, and as black spots in 2*b*, according to focusing. (Exposure: 48 days and development time: 2 min. in D-19b.)

The random errors, as distinct from the systematic ones mentioned above, and observer bias, arise from the necessity of counting relatively small numbers of cells (sampling error) and from the fact that the background correction for samples which have a large number of unlabeled cells yields a big correction to the observed cells with one grain.

The probability that random sampling errors will give rise to a non-Poisson distribution may be estimated by use of the chi square test. In this test one assumes that the Poisson distribution, using the measured value of $\bar{g}$, is correct and calculates, from the observed deviations from this distribution, the probability P that random fluctuations could have given rise to the observed distribution. The Poisson model is accepted if P falls between 0.05 and 0.95. Such a procedure ignores systematic errors. Some of the distributions for uridine, leucine, and DAP differ slightly, but significantly, after 7 or more generations, from a Poisson. This probably indicates a systematic error such as latent-image fading, track formation, dis-

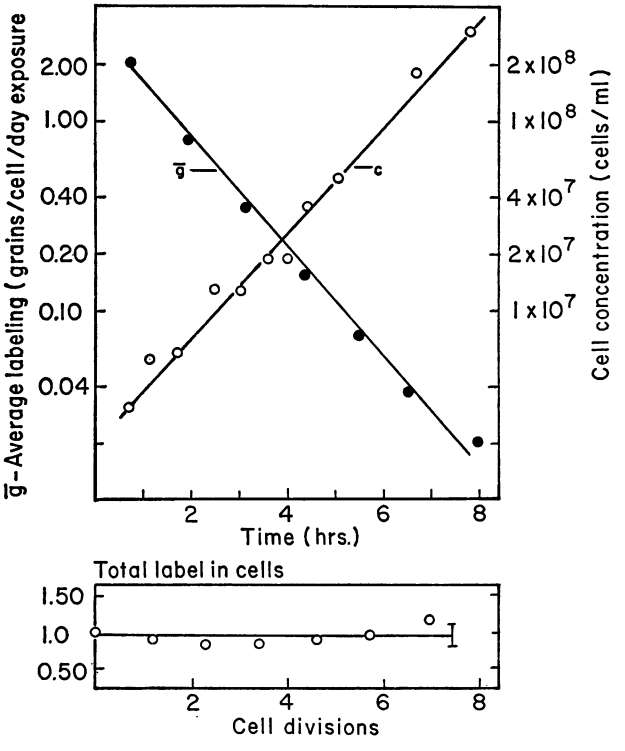

FIGURE 3 The upper section shows, on a logarithmic scale, the average grain count, $\bar{g}$, and the cell concentration, c, plotted *versus* time for H^3DAP in *E. coli* 173:25: DAP^-. The relative product of the two sets of data for different division times is shown in the lower section.

tribution of division times, etc. Others differ so drastically that systematic errors cannot account for the results and we interpret them in terms of the model of Fig. 1*b*. To assist the reader in forming his own judgment we have given, in the figure legends, the probability P that random errors account for the differences between observed and Poisson distributions. The method of computing these probabilities is similar to that used by Caro (35).

II. *Cell Wall*

A. *Evidence for Label in Cell Wall.* DAP[1] has been found to be a component of cell wall protein (24, 25). Its vital role in cellular integrity has been demonstrated (26-30). A labeled racemic mixture (California Biochemical Foundation) was used to satisfy the requirements of *E. coli* 173:25:DAP$^-$. This strain has a relative requirement for L-lysine and since the strain has the enzyme DAP-decarboxylase (31) and DAP is an intermediate in lysine metabolism (9), the labeling was carried out in the presence of ~200 to 1 excess of unlabeled lysine so as to minimize the appearance of H^3-lysine in protein.

The experimental justification for associating the radioactive label with cell wall material is as follows:—

1. Cells labeled with DAP were treated with chymotrypsin before autography. Less than 12 per cent of the label was removed whereas similar enzymatic treatment removed 70 per cent of H^3-proline and H^3-leucine labels. Chymotrypsin-digested cells appeared as faint "ghosts" in phase contrast. The ghosts were pre-

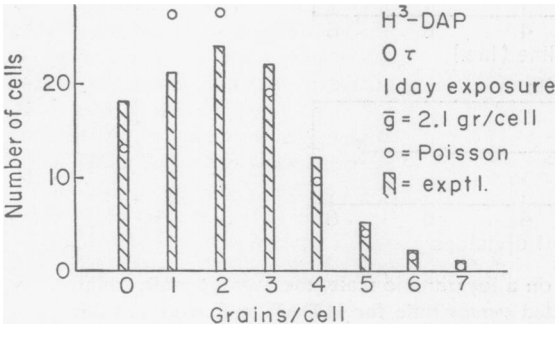

FIGURE 4*a*

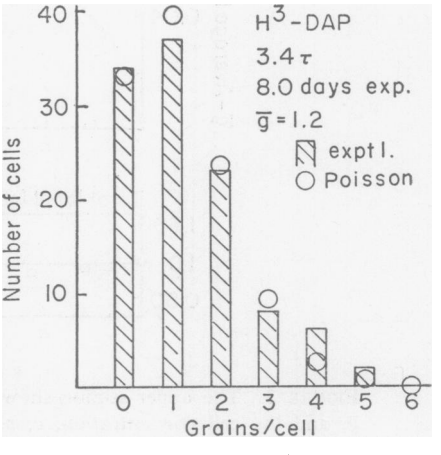

FIGURE 4*b*

sumably the cell walls and they were labeled. Only 30 per cent of the label on planchettes was removed by chymotrypsin.

2. Labeled cells deprived of DAP and grown in 5 per cent sucrose medium were made into large protoplasts (30) and, before lysis, were fixed in 5 per cent formaldehyde, washed and autographed. The grain count for the fully developed protoplasts was less than 10 per cent that of the original cells. Fig. 2 shows radioautographs of intact cells, emerging protoplasts, and mature protoplasts labeled with H^3 DAP. The attenuated cell wall was frequently identified in debris near the protoplasts, and this indicates that these walls were labeled. Petroff-Hauser chamber counts showed that, in this experiment, cell growth was less than a factor of two. Hence >80 per cent of the label was lost by cells becoming protoplasts. The remaining <20 per cent is probably in protein or in cell wall material not lost by the protoplasts.

3. Cells were grown to 8×10^8/ml and 0.9 ml, spun down, and resuspended in water 4 times. The amount of radioactivity in the suspension which was not associated with cells was about 0.2 per cent of that in the cells. The cells were hydrolyzed at 100°C in 6 N HCl for 18 hrs (32, 33). The hydrolysate was dried over KOH, resuspended in 0.11 ml H_2O and 0.01 ml was chromatographed as indicated in Materials and Methods. The activity as a function of position was determined with a gas flow counter. The ratio of activities in the DAP spot to the lysine spot

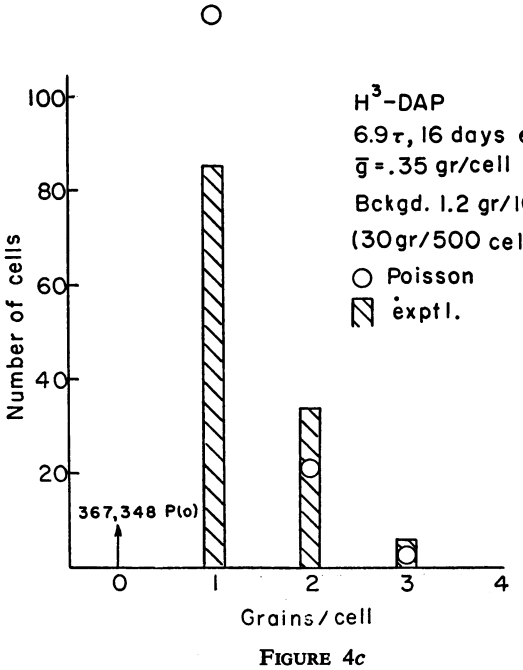

FIGURE 4c

FIGURE 4

Observed grain distribution curves for H^3DAP in *E. coli* 173:25DAP⁻. The Poisson distribution is calculated from the observed average grain count, $\bar{g}$, indicated on the figures. (*a*) 0τ, (*b*) 3.4τ, (*c*) 6.9τ. For 1 grain/cell, a maximum background correction of 30 cells was subtracted from the 1 grain column for the 6.9τ data to give the value shown in the figure. [Chi square test: 4*a*, $P > 0.05$; 4*b*, $P > 0.05$; 4*c*, $P < 0.05$ (~ 0.01).]

was 6 to 1. Thus at least 85 per cent of the label is in the cell wall. Other methods of growth and labeling give a much smaller fraction in the cell wall (33).

4. H^3DAP quantitatively satisfies the growth requirement of the cells.

5. Unlabeled DAP competes with labeled DAP for incorporation into cells.

B. *Conservation of Cell Wall Material.* Fig. 3 shows the average cellular grain count and the cellular growth as a function of time for initially fully labeled cells growing in medium containing unlabeled DAP. The reciprocal relationship between growth and $\bar{g}$ indicates that the label is conserved in the cells.

C. *Distribution of Cell Wall Material among Progeny.* Grain distributions for two experiments are shown in Figs. 4 and 5. The Poisson distributions calculated from the values of $\bar{g}$ are also indicated. The agreement between the calculated and observed distributions is excellent until the seventh and eight generations. The departures at 8.3τ and 6.9τ are definitely significant but are in no sense comparable to those observed for DNA inheritance. The departure from Poisson is explicable if about 10 per cent of the β-rays entering the emulsion give rise to 2 grains. (Such an effect would not be noticed at higher values of $\bar{g}$.)

The general result indicated by these data is that the H^3DAP label is not in large, intact structures. If we were to choose between schemes (*a*) and (*b*) of

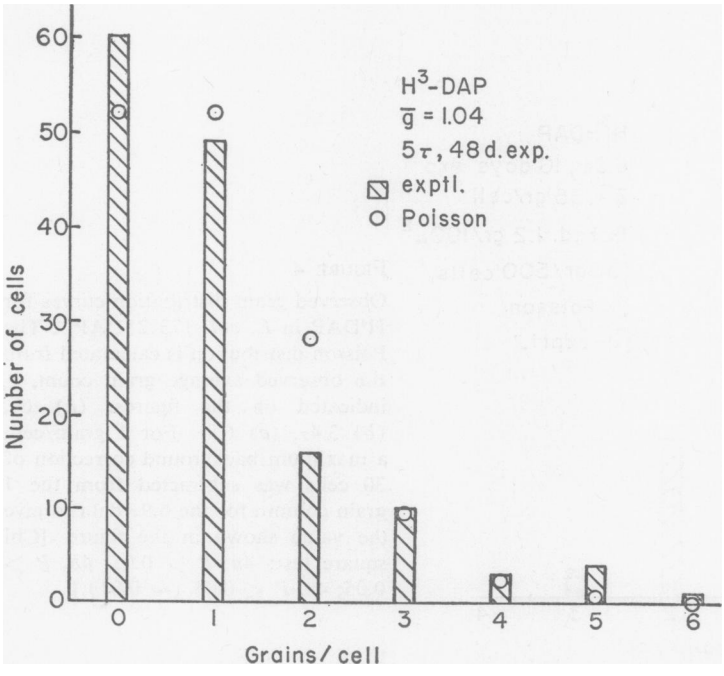

FIGURE *5a*

Fig. 1, we would choose the random case (*a*) through the 8th generation. Thus the number of cell wall units is greater than 200.

III. *RNA*

A. *Evidence for Label in RNA.* H^3U (34) was used to label RNA in 15T⁻U⁻ and 15T⁻U⁻A⁻. Caro (35) has shown that about 90 per cent of this label is found in the RNA fraction of these cells, the remainder being in DNA. H^3U competes with unlabeled uridine and it satisfies the auxotrophic requirement of these cells.

B. *Conservation of RNA.* A summary of radioautograph and cell concentration data carried out for 3 generations on 15T⁻U⁻ is shown in the lower part of Fig. 6. The data indicate no loss of label. Geiger counter data obtained on 8 generations of exponentially growing cells indicate that, within the experimental error of 15 per cent, the label is conserved. Davern and Meselson (36) have found by density-gradient centrifugation that in 3τ there is no measurable turnover of intracellular RNA.

C. *Distribution of RNA among Progeny.* The upper part of Fig. 6 shows the distribution of grain counts for 15T⁻U⁻ over a period of 3τ. The distributions

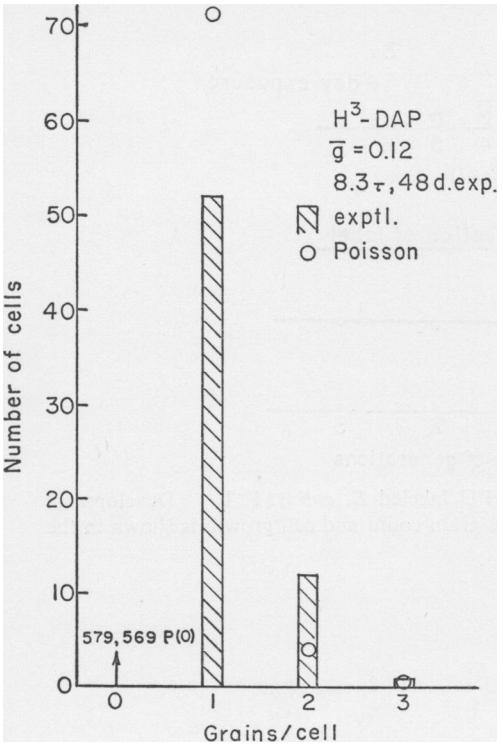

FIGURE 5b

FIGURE 5

Grain distribution curves for H^3DAP in *E. coli* 173:25:DAP⁻. The exposure was carried out in a CO_2 atmosphere to minimize fading. The low background grain count, 0.2 gr/100 μ^2, gave negligible correction to the observed data. (Development time: 2 min. in D-19b). (*a*) 5τ, (*b*) 8.3τ. [Chi square test: *5a*, $P < 0.05$ ($\sim$ .02) *5b*, $P < 0.05$]

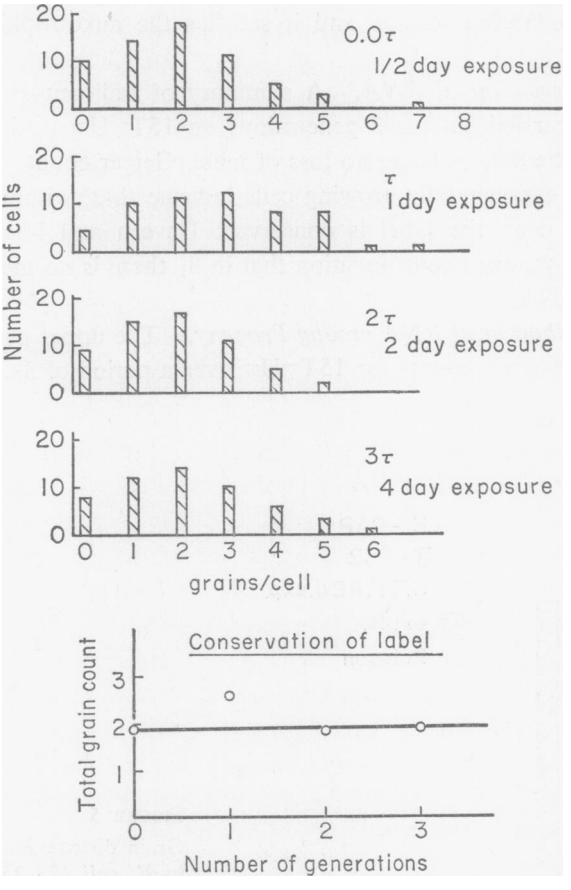

FIGURE 6 Grain distribution data for H^3U labeled *E. coli* $15T^-U^-$ (Development time: 8 min.) and the product of average grain count and cell growth is shown in the lower section.

are obviously random and show, after normalization to similar exposure times, that $\bar{g} = \bar{g}_0/2^k$. The distribution curves for 15T⁻U⁻ at 4τ, 7τ, and 8τ obtained in a separate experiment are shown in Fig. 7. There is excellent agreement between observation and the Poisson distribution calculated from $\bar{g}$. The deviation at 8τ could result from about 5 per cent of the H^3U label appearing in DNA (35). The DNA units are small in number and we would not expect the DNA label to be distributed at random (see below); alternatively the deviations could, as mentioned in connection with DAP, arise from a β-particle giving two grains. At 8τ the culture was approaching the lag phase of growth and the deviations might represent about 5 per cent of the cells not dividing between 7τ and 8τ.

Data similar to the above are shown in Fig. 8 for the distribution of H^3U among progeny of 15T⁻U⁻A⁻. Again we see that the distributions are near random for all generations.

We conclude that there must be more than 200 RNA units in *E. coli*. This number is less than 20,000 (56), the estimated number of ribosomes per cell.

IV. *Protein*

A. *Evidence for Label in Protein.* The proteins in 15T⁻U⁻ and 15-T⁻U⁻A⁻ were labeled with H^3-proline (15) and H^3-DL-leucine (15, 37). H^3-proline quantitatively satisfies the auxotrophic requirement of the prolineless mutant *E. coli* W-55-1. Both tritiated compounds compete with the similar unlabeled L-amino acids for incorporation into cells. Only the L-amino acid was incorporated from medium containing H^3-DL-leucine as shown by the observations that only L-leucine competes for the uptake of label from H^3-DL-leucine and that the cells, at most, take up half the total available label. The label, as measured by a Geiger counter, in cells grown in H^3-DL-leucine can be removed by chymotrypsin but not by DNase or RNase as is indicated in Fig. 9. Similar results were obtained with radioautographic observation.

B. *Conservation of Protein.* Geiger counter and radioautographic data (not shown) on 15T⁻U⁻ indicate that, within the experimental error of 15 per cent, the H^3-leucine label is conserved for 8 generations during exponential growth and that, within an error of 10 per cent, the H^3-proline label in 15T⁻U⁻A⁻ is conserved for at least 5 generations.

C. *Distribution of Protein among Progeny.* The grain distribution data for 15T⁻U⁻A⁻ labeled with H^3-proline are shown in Fig. 10. Up to 4.6τ the data fit, to within experimental error, the Poisson distribution calculated from $\bar{g}$. Similar distribution data are shown in Fig. 11 for H^3-leucine in 15T⁻U⁻A⁻ for times up to 4τ. Again the data fit Poisson distributions.

The distribution for 8τ for 15T⁻U⁻ labeled with H^3-leucine is given in Fig. 12. The distribution shows a small but significant (by chi square test) deviation from a Poisson. The first few division times, in this experiment, were characterized by

an appreciable fraction of the cells elongating but not dividing. At the time that the radioautographs were made, all cells, however, were of normal length. The departures from a Poisson distribution may have arisen from this fact or from a large spread in division times in this particular experiment.

The general conclusion is that proteins are distributed randomly to progeny cells for at least 7 to 8 generations. There are thus more than 2^7 to 2^8 (~200) protein units per cell. This is not too surprising a result since a bacterium contains of the order of 10^6 protein molecules.

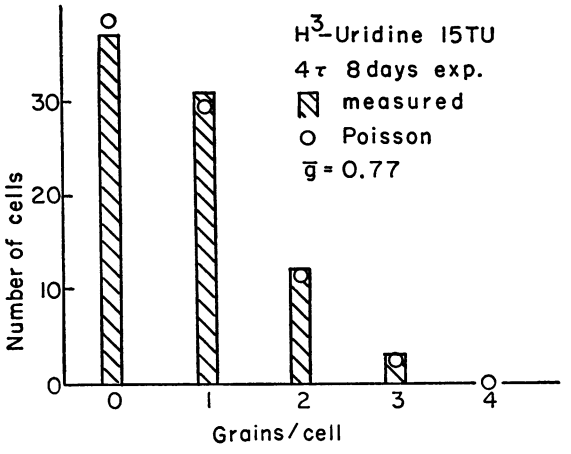

FIGURE 7*a*

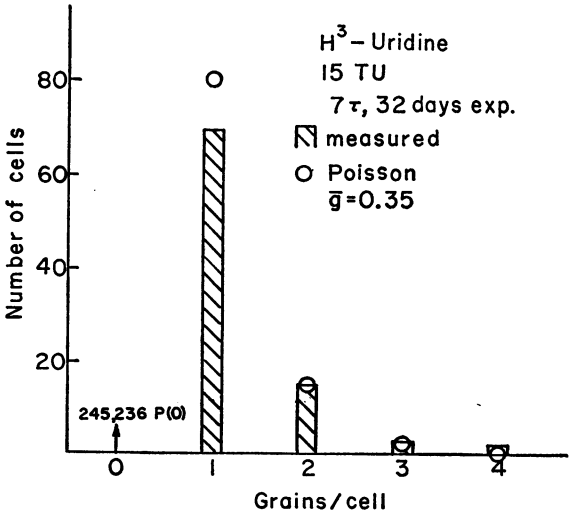

FIGURE 7*b*

V. *DNA*

A. *Evidence for Label in DNA.* Cells of 15T⁻ and 15T⁻U⁻ were labeled with H^3T (1). There is ample chemical evidence that H^3T appears in DNA (38-40). The demonstration that H^3T is localized in chromosomes and chromosome-like units is found in the work of many investigators (41-43, 1, 2). The role of thymidine as a nuclear component has been demonstrated (44-47), and Caro *et al.* (48) have localized H^3T in the nuclear region of *E. coli* 15T⁻. Our experimental reasons for associating incorporated H^3T with cellular DNA are:

1. Unlabeled thymidine competes with H^3T for incorporation into 15T⁻ and 15T⁻U⁻.
2. H^3T quantitatively satisfies the growth requirements of these strains.
3. The incorporated label is susceptible to the action of DNase but not to RNase, trypsin or chymotrypsin[2] (17, 18, 42, 49-51). Typical enzymatic treatment data are shown in Fig. 13 for samples on planchettes. Similar results were obtained with radioautographs.

B. *Conservation of DNA.* An experiment which shows that no label is lost from 15T⁻U⁻ during exponential growth is indicated in Fig. 14. The cell concentration, as measured by Petroff-Hauser counts, increases exponentially with time while the average grain count, $\bar{g}$, decreases exponentially with time. As shown

[2] Cells must be pretreated with 2 per cent formaldehyde if the labeled DNA is not to be removed by protease digestion.

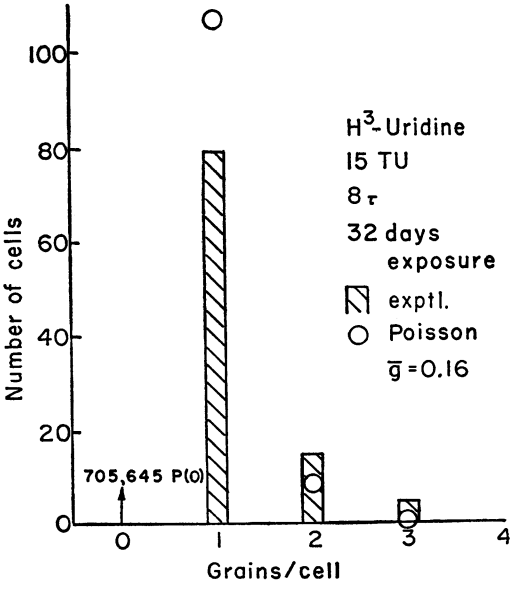

FIGURE 7c

FIGURE 7

Grain distribution curves for H^3U in *E. coli* 15T⁻U⁻. The exposure was carried out in a CO_2 atmosphere. (Development time: 2 min in D-19b). (*a*) 4τ, (*b*) 7τ and (*c*) 8τ. (705 cells with zero grains; expected from Poisson, 645.) [Chi square test: 7*a*, $P > 0.05$; 7*b*, $P > 0.05$; 7*c*, $P < 0.05$ (~ 0.01).]

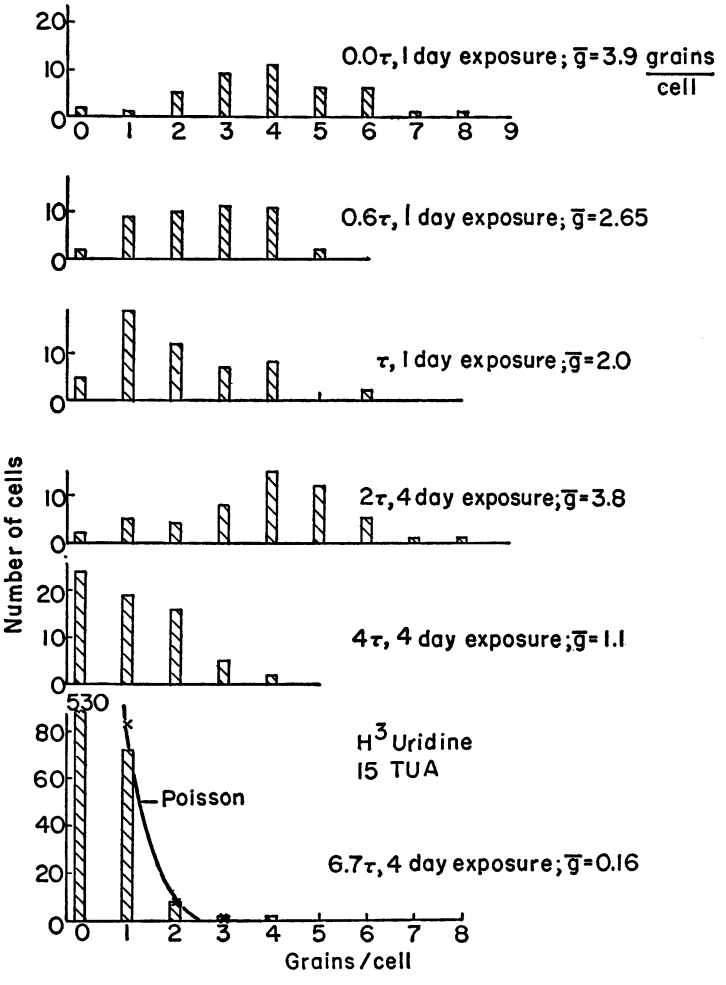

FIGURE 8 Grain distribution data for H^3U in *E. coli* $15T^-U^-A^-$.

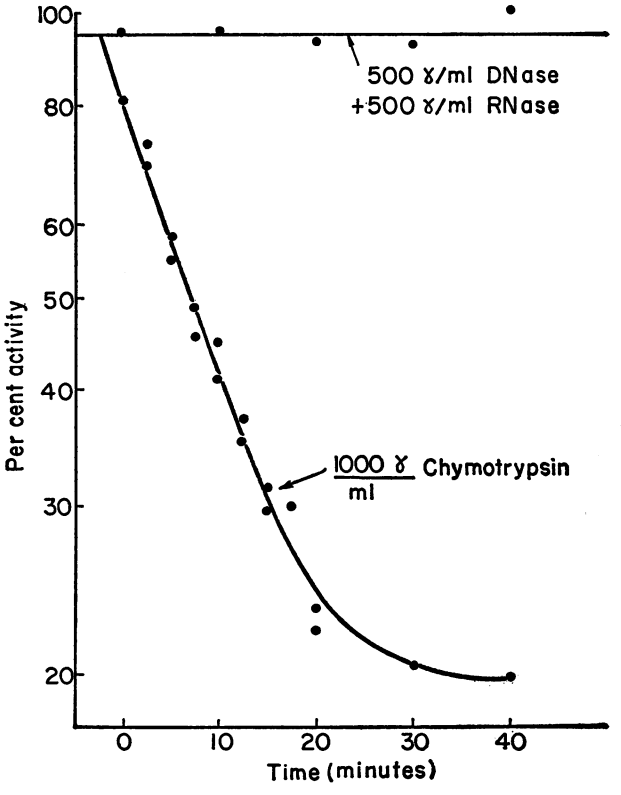

FIGURE 9 Enzymatic removal of H^3-leucine in *E. coli* 15T⁻U⁻A⁻ cells on planchettes. Geiger counts are plotted *versus* time of treatment. The ordinate is reduced by 5 per cent by the buffer treatment alone.

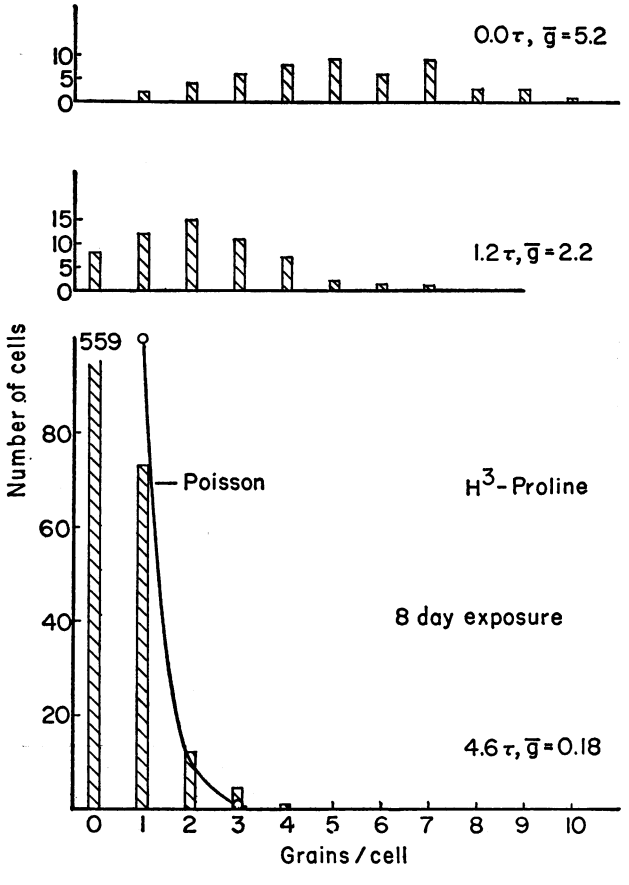

FIGURE 10 Grain count distribution data for H^3-proline in *E. coli* 15T⁻U⁻A⁻.

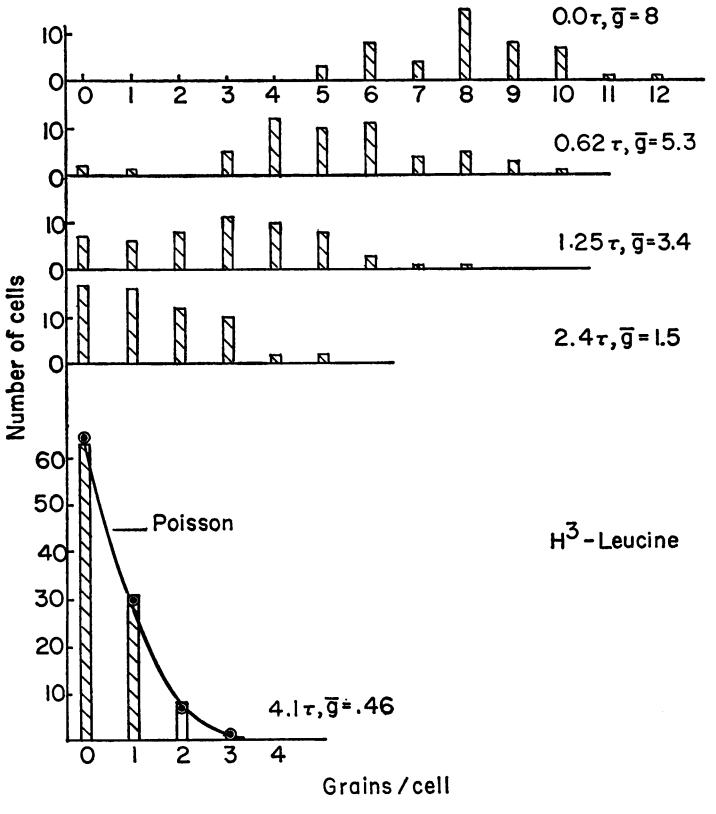

FIGURE 11 Grain count distribution data for H^3-leucine in *E. coli* 15T⁻U⁻A⁻. (Exposure: ½ day.)

in the lower part of Fig. 14, the product of cell concentraion and $\bar{g}$ is consant for 5 generations. As found by Hershey (52), there is no loss of label. This finding was checked by Geiger counter data. Fig. 15 shows typical data for 15T⁻ grown at 37°C with aeration. The counting rate per 0.01 ml of cells is constant for 7τ. The label is conserved. Similar data for 15T⁻U⁻ showed conservation of DNA label for 8 generations of exponential growth at 23.5°C.

C. *Distribution of DNA among Progeny.* A growth curve and photographs of radioautographs at 2τ, 3τ, and 4τ in a typical experiment on 15T⁻ are shown in Fig. 16. The estimated uncertainty in the times of radioautographs is no more than $\pm 0.1\tau$ up to 3τ and $\pm 0.2\tau$ for 4τ. It should be apparent that at 2τ practically *all* the cells are labeled. Quantitative measurements indicate that at this

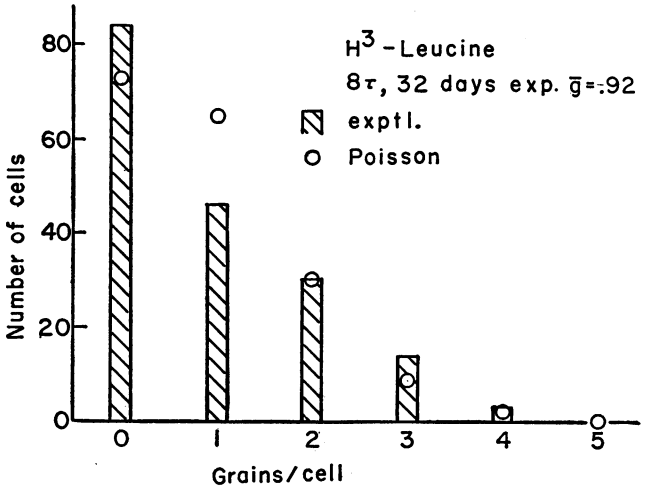

FIGURE 12 The observed grain distribution curve for H^3-leucine in *E. coli* 15T⁻U⁻ at the eighth generation. The exposure was carried out in a CO_2 atmosphere. (Development time: 2 min in D-19b). [Chi square test: $P < 0.05$ (~ 0.01)]

time $\bar{g} = 4$ grains/cell and that the fraction of cells with no grains is <2 per cent. This result is expected in a Poisson distribution because $P(0) = e^{-4} = 0.018$. Note that after 3 or more generations a sizeable fraction of the cells show no grains. These fractions, about 1/3 for 3τ and 2/3 for 4τ, would not be expected from a simple Poisson distribution. At 3τ, we have $\bar{g} = 2$ and $P(0) = e^{-2} = 0.14$ while at 4τ, $\bar{g} = 1$ and $P(0) = 0.37$. The marked deviation from a Poisson distribution which occurs abruptly at 3τ suggests that we are dealing with a non-random process of the type illustrated in Fig. 1*b* in which the number of units is $2^{(3-1)} = 4$. The further analysis of these data is best given in terms of a comparison of the observed and calculated distribution functions.

The grain distribution data from an experiment on 15T⁻ are shown in Fig. 17

and those from an experiment on 15T—U— are shown in Fig. 18. Even though the numbers of cells measured in these experiments was small, a cursory inspection of the data shows that the distributions are close to random through 2τ and that the number of unlabeled cells increases markedly at 3τ. The results of an experiment designed to investigate this abrupt transition more quantitatively for 15T— are shown in Fig. 19. The data for 2τ have been adjusted to give the same total grain count as for 3τ. To a first approximation the 2τ data fit a Poisson distribution and the 3τ data definitely do not. According to our model this suggests that there are at least 4 non-randomly distributed units. On the other hand, if there were only 4 units to be reckoned with, there would not be so many cells at 3τ with 1 and 2 grains/cell. The abrupt large asymmetry at 3τ allows us to rule out cells with only 8 randomly segregating structures,[3] but we must turn to the distributions of later

[3] At 2τ we would expect 8 random units to be distributed among the 4 cells so that the probability of a cell containing no units would be $(\frac{3}{4})^8 = 10$ per cent. Fig. 19 shows much less than 10 per cent unlabeled cells at 2τ.

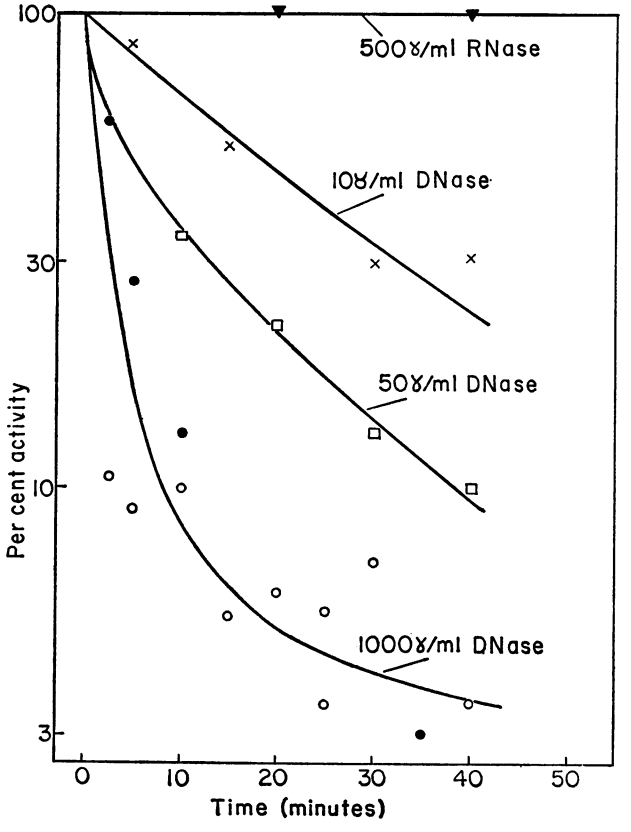

FIGURE 13 Enzymatic removal of H^3T label in *E. coli* 15T— cells placed on planchettes. Relative Geiger counter data are plotted *versus* time of treatment.

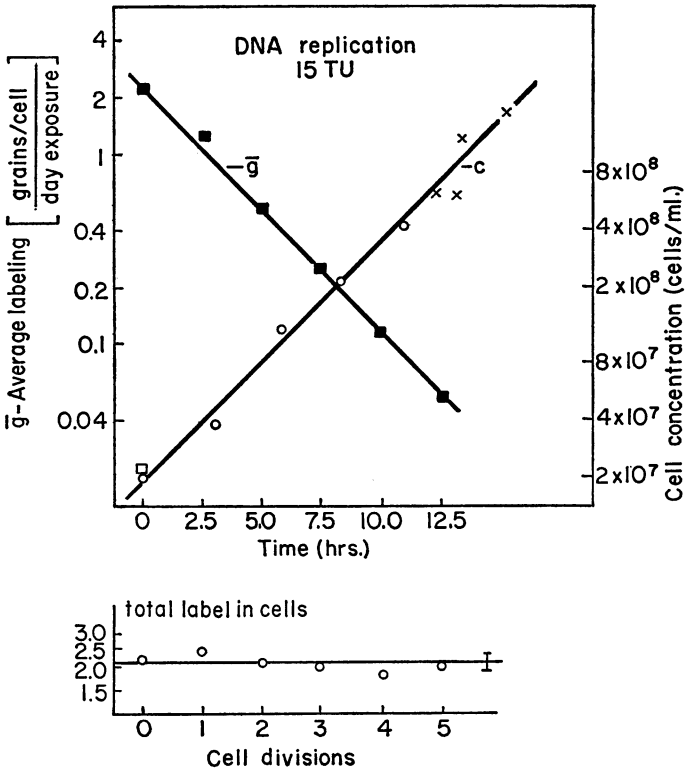

FIGURE 14 The conservation of H^3T in cells of *E. coli* $15T^-U^-$ subsequently deprived of H^3T. Average grain count and the cell concentration *c*, both on logarithmic scales plotted *versus* time. The relative product of the two sets of data for different division times is plotted in the lower section.

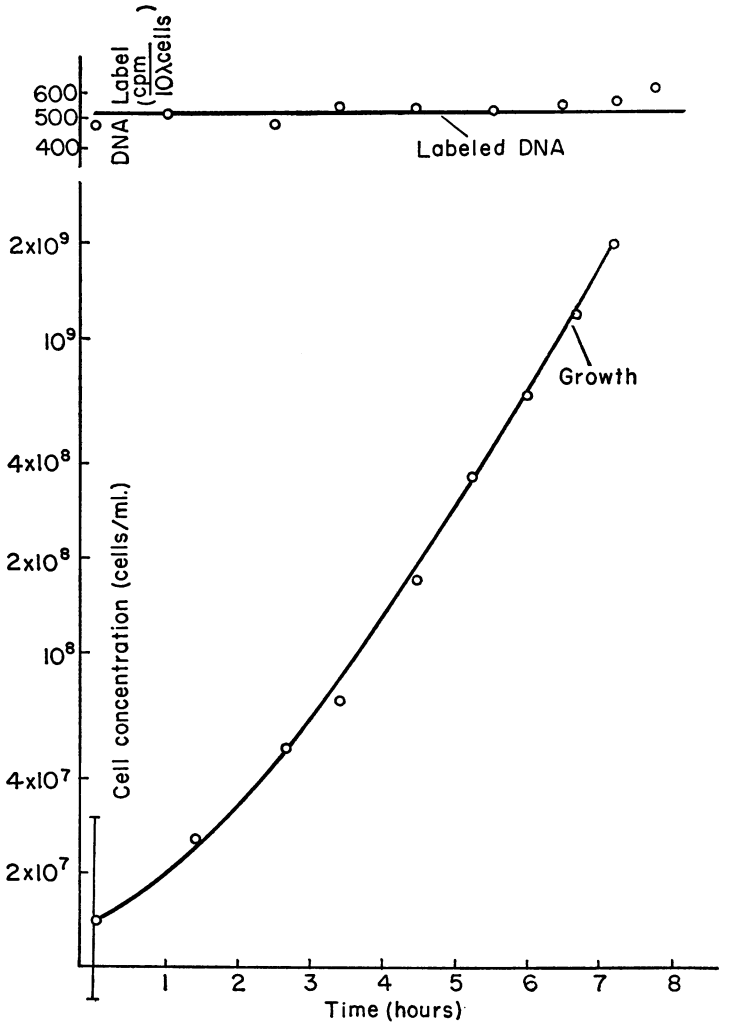

FIGURE 15 Geiger count data for H^3T in cells of *E. coli* $15T^-$ subsequently deprived of H^3T. The upper curve shows the Geiger counts for constant volumes of cells. The lower curve shows the population growth.

generations for a more precise analysis. Fig. 20 shows data on 15T⁻ for times up to 5τ with a reasonably high grain count in the labeled cells. These distributions which have not been normalized to the same total grain count indicate qualitatively that the shape of the distribution curves changes little between 2τ and 5τ, except for the abrupt increases in the number of cells with zero and 1 grain at 3τ. These data indicate that many of the cells have 4 non-random units and others may have 8 labeled units, all of which are not the same size (see section D). The stability of these supposed units was investigated in an experiment comparing a population of cells slightly beyond 2τ with a 7τ population. In the data shown in Fig. 21 the 2τ data have been adjusted to the same total grain count as for 7τ. The intact nature of the individual DNA structures is clearly seen. Despite appreciable latent-image fading in this experiment the two distributions coincide except for the differences at low grain counts which we ascribed above to units other than the 4 large non-random ones. The data of Fig. 21 imply a remarkable stability of these structures.

An experiment on 15T⁻ was performed for the purpose of investigating more critically the stability of the DNA units. The experimental details differed in three respects from those given in Materials and Methods. A higher rated specific activity (2.6 c/mM) H^3T was used so as to permit smaller exposure times and hence less latent image fading. Both 0τ and 7τ cultures were fixed in 0.02 per cent formalin. The fixed cells were centrifuged and resuspended to remove any H^3T in the medium, sonicated gently (a procedure which reduced cell clumping) and then placed on glass slides and dried. The omission of Carnoy's solution from the procedure seemed to result in a better morphological preservation of the cells. A 1 minute

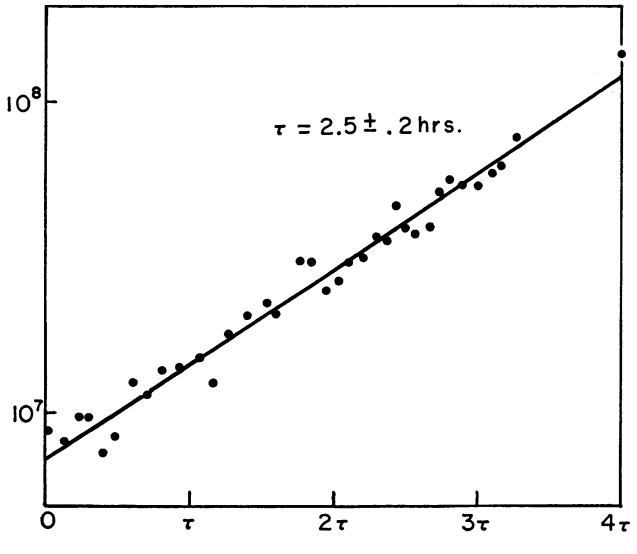

FIGURE 16*d*
The growth curve of the culture. The time of removal of the cells for radioautography is denoted by integral units of τ.

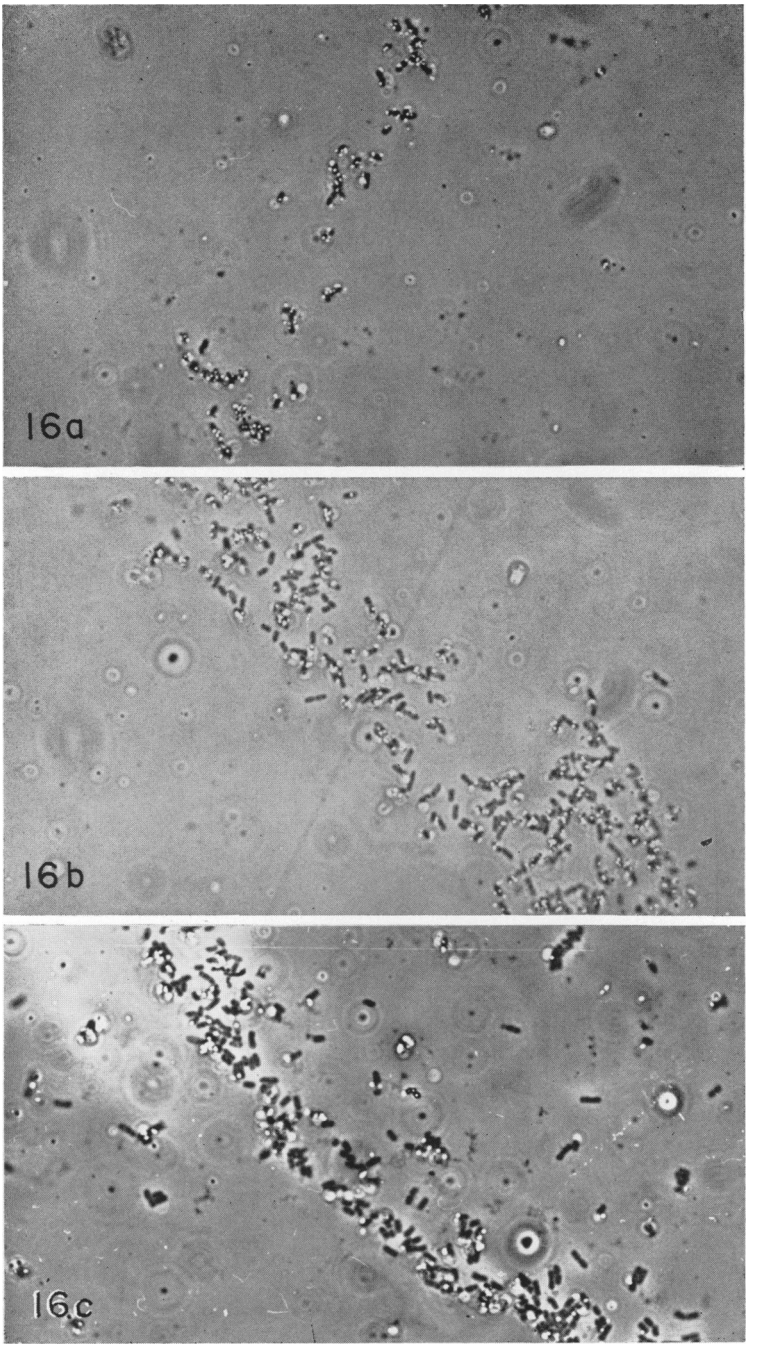

FIGURE 16*a-c* Phase contrast photographs of radioautographs of H^3T labeled *E. coli* $15T^-$ at various times. (*a*) 2τ, $\bar{g} = 4$ grains/cell, (*b*) 3τ, (*c*) 4τ. Latent-image fading was about 30 per cent. (Exposure: 8 days.)

development time was used to yield very small, easily resolvable grains and low background. The high resolution was necessary because by 7τ many large DNA units are in one half of a cell (53) and the resulting grains closer together than for fully labeled cells. Reichert phase and bright field optics were used. The grain distributions for 0τ and 7τ are shown in Fig. 22. The data, as in previous experiments, deviate at low grain counts from a Poisson distribution calculated assuming only 4 large units.

D. *Quantitative Analysis of the Distribution of DNA among Progeny Cells.* All cells at 0τ, 1τ, and 2τ have labeled DNA. The number of cells which show zero grains is just that expected from the Poisson distribution. The evidence for this statement is given in Table IV which shows a comparison of the observed zero-grain frequency and the calculated frequency, $P(0) = e^{-\bar{g}}$, for 10 separate experiments.

We have pointed out that the abrupt change in the distribution at 3τ implies the existence of a sizeable fraction of cells with 4 large non-randomly distributed structures. However, if all the DNA were in these units, 50 per cent of the cells at 3τ would contain no radioactive DNA. But because of the random nature of radioactive decay some of the labeled cells will not show grains and thus the number of cells showing no grains will be greater than 50 per cent. In actual fact the number of zero-grain cells is *less* than 50 per cent. For example, in Fig. 19 the fraction of observed zero-grain cells is 0.39. An obvious possibility is that some cells have more than 4 units, and, on this model, the observed distributions are the sum of several Poisson distributions. Each distribution would represent cells which originally had 4, 8, 16 . . . units per cell. Our data are not good enough to detect anything but units in multiples of 2 and besides there are good theoretical reasons for choosing this multiple.

The procedure followed for resolving the observed distributions into component Poisson distributions follows. We rewrite the Poisson distribution in the form

$$n!P(n) = \bar{g}^n e^{-\bar{g}}$$

and take the logarithm of both sides

$$\ln [n!P(n)] = n \ln \bar{g} - \bar{g}$$

Thus a plot of $\ln [n!P(n)]$ *versus* n should be a straight line[4] of slope $\ln \bar{g}$ and intercept $-\bar{g}$. If the actual distribution is the sum of two Poissons, the plot would be the sum of two straight lines. In actual practice we have plotted not $\ln [n!P(n)]$ but $\log [n! \cdot \text{number } (n)]$ which is permissible since the two functions are proportional to one another. Fig. 23 shows the 3τ data of Fig. 19 plotted in this fashion. If the straight line representing large values of n (a Poisson distribution representing large units with $\bar{g} = \bar{g}_l$) is extrapolated to low values of n and the difference

[4] We are indebted to Dr. P. C. Hanawalt for pointing this out to us.

TABLE IV

A COMPARISON OF THE OBSERVED AND CALCULATED FREQUENCIES OF CELLS WITH ZERO GRAINS FOR H^3T LABELING

Cells	Time	$\bar{g}$ (gr/cell)	$P(0)$ Calculated	$P(0)$ Observed
			per cent	*per cent*
$15T^-$	0τ	6.2	<0.2	<0.3
	1τ	2.9	6	2
	2τ	1.6	20	23
	2τ	4.9	0.7	1.1
	2τ	4.7	1.0	4.5
	2τ	4.0	1.8	2.0
at 37°C	2τ	2.3	10	7
$15T^-U^-$	0τ	2.2	11	12
	1τ	4.9	0.7	2
	2τ	2.0	12	17

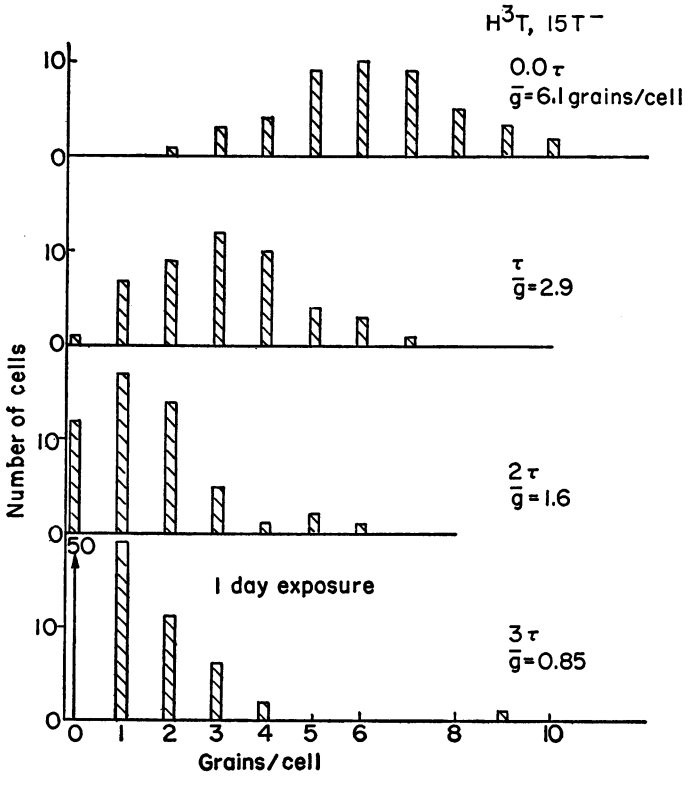

FIGURE 17 Grain count data for H^3T in *E. coli* $15T^-$ (Exposure: 1 day, development time: 8 minutes).

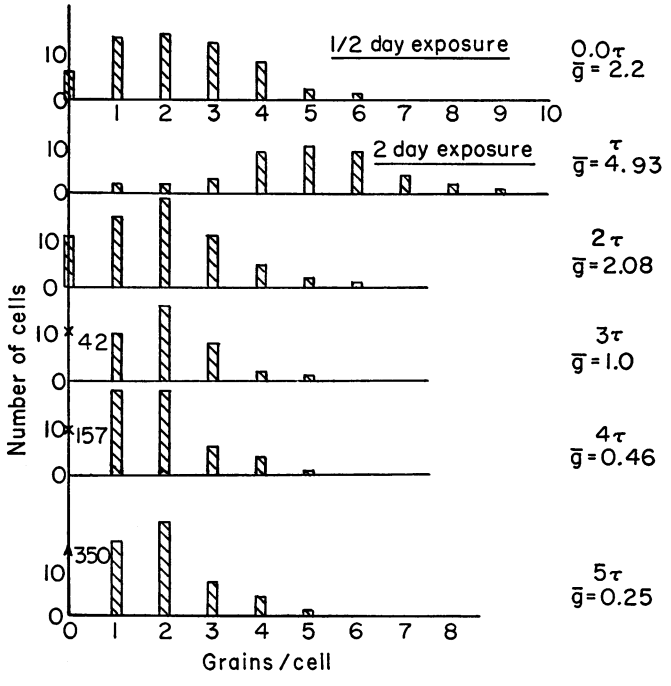

FIGURE 18 Grain count data for H³T in *E. coli* 15T⁻U⁻.

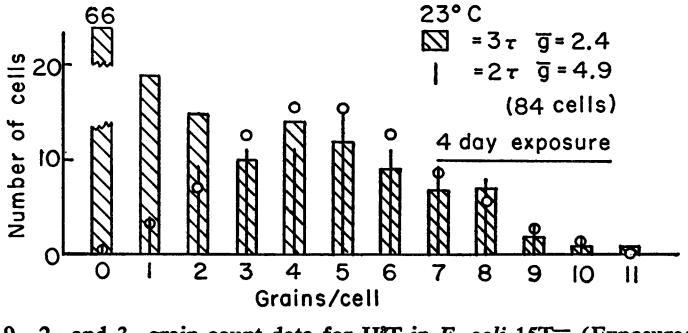

FIGURE 19 2τ and 3τ grain count data for H³T in *E. coli* 15T⁻ (Exposure: 4 days, development time: 8 min.) [Chi square test: 2τ, $P > 0.05$; 3τ, $P \ll 0.005$.]

between it and the observed values computed, the points represented by crosses are obtained. If now a straight line (a Poisson distribution representing small units with $\bar{g} = \bar{g}_s$) is drawn through points $n = 1, 2, 3, 4$ (the values at n = 3, 4 being uncertain) and its intercept subtracted from the observed value for $n = 0$ we obtain directly the number of cells for which $\bar{g} = 0$. The total numbers of cells associated with $\bar{g}_s$ and $\bar{g}_l$ may be found from the straight lines representing these distributions. In this way we find the fraction of cells which have small units. From the data of Fig. 23, $\bar{g}_s = 1.0$ and $\bar{g}_l = 5.4$ grains/cell and the fraction of labeled cells with small units is 0.4.

The resolution of the observed 3τ distribution into three Poissons with values

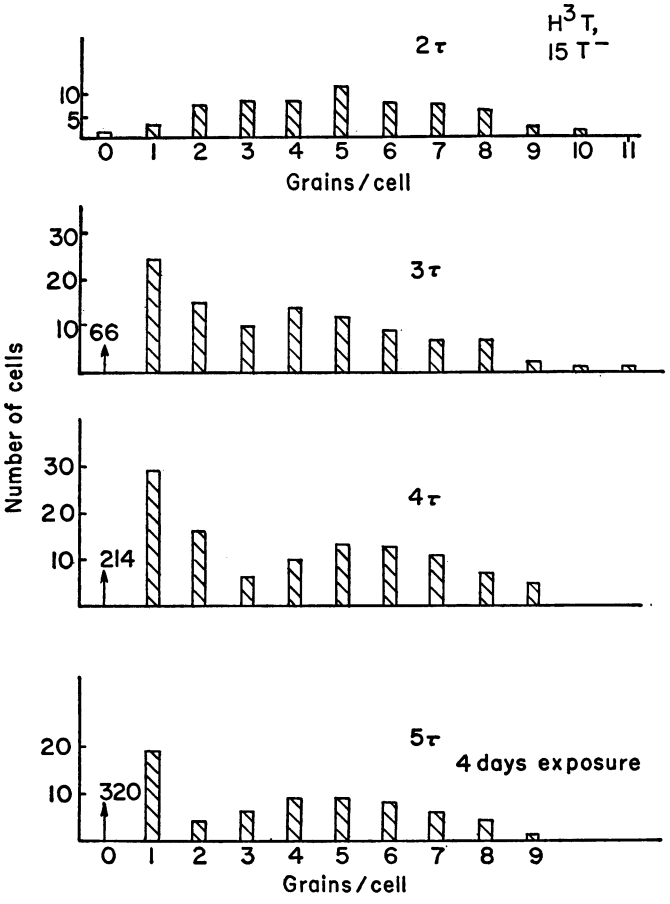

FIGURE 20 2τ, 3τ, 4τ, and 5τ grain count data for H^3T in *E. coli* 15T⁻ (Exposure: 4 days, development time: 8 min.). 2τ, 3τ data are the same as in Fig. 19. [Chi square test: 2τ, $P > 0.05$; all others, $P \ll 0.005$.]

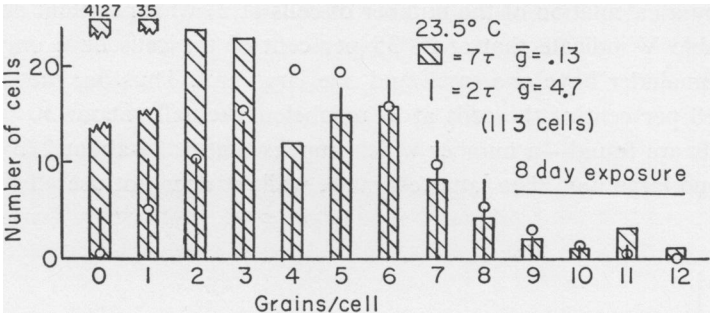

FIGURE 21 2τ, and 7τ grain count data for H^3T in *E. coli* 15T⁻ (Exposure: 8 days; about 30 per cent latent-image fading). The 7τ cells were packed so that each cell occupies about $2\mu^2$. Using this information we calculate a minimum correction of about 55 grains due to background, and this number has been subtracted from the 1 grain column. [Chi square test: 0τ, $P > 0.05$; 7τ, $P \ll 0.005$.]

of $\bar{g} = 0$, $\bar{g}_s$, and $\bar{g}_l$ implies that the cells with $\bar{g} = 0$ arise from the division at 3τ of cells with only 4 large units. It is worth pointing out that the distribution for the small units is subject to very large uncertainties because it is determined mostly by the data of $n = 1, 2$, whereas those for $\bar{g} = 0$ and $\bar{g}_l$ are not so uncertain. The values at $n = 1$ may have large corrections due to background (see Figs. 21 and 22) and the values at $n = 2$ have small-number sampling fluctuations which are not present at $n = 0$ or for the average of n = 3, 4, 5, ... Fig. 24 shows a similar treatment of the data for the 7τ distribution shown in Fig. 21. The resolution into 3 Poisson distributions is apparent. The values obtained are $\bar{g}_s = 1.1$, $\bar{g}_l = 5.0$ and the fraction of labeled cells which have small units is 0.5. A summary of the data analyzed this way is shown in Table V.

Within experimental error, the fraction of labeled cells with small units is constant in time from 3τ to 7τ and is 0.40. The size is about 1/5 that of the large units. Thus we would describe the populations of 0τ cells as made up of 35 per cent cells with 4 large units of DNA and 65 per cent with 4 large units plus 4 small units of DNA. Since at 3τ 40 per cent of the labeled cells have small units and each unit is approximately 1/5 the size of the large units, approximately 10 per cent of the total radioactivity is in the smaller units. This figure represents an upper limit for the amount of DNA which is randomly distributed to progeny cells.

E. *Further Remarks on DNA Distribution among Progeny Cells.* We give below additional information and estimates on the number of DNA units per cell and estimate the stability of the large units. The calculations give only estimates because not only is the starting population heterogeneous in size but the resolution of the Poisson distribution (at $\bar{g} > 4.5$) is not sufficient to see the difference between units of size one and the average of equal numbers of units of sizes 2/3 and 4/3. The averages for $\bar{g}$ are indeed grand averages. The poor resolution is best

indicated by a calculation of the number of cells at 3τ which contain no label. The data of Table V indicate that at 2τ 35 per cent of the cells have only one large unit, the remainder have one small and one large unit. Thus one would expect to find only 18 per cent of the cells at 3τ unlabeled. Actually about 30 per cent unlabeled cells are found—a number which implies that at 2τ about 2/3 of the cells had only one large unit. The latter estimate, while it does not use all the available

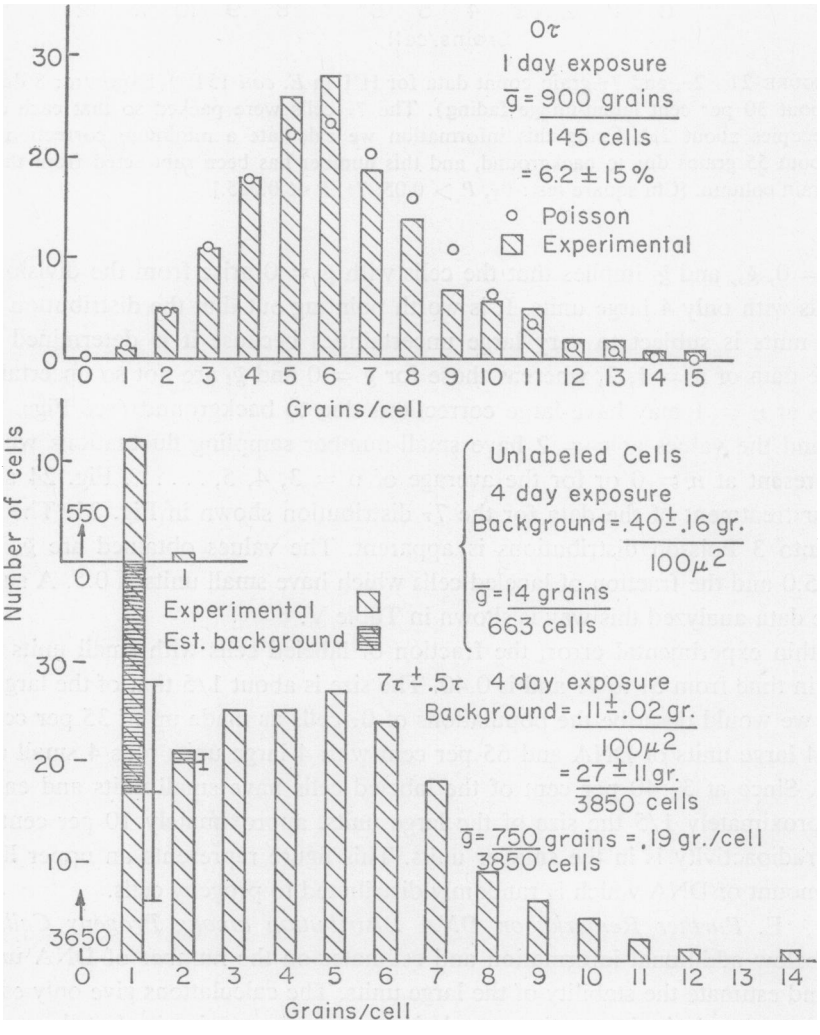

FIGURE 22*a* 0τ, unlabeled cells and 7τ grain count data for H^3T (2.6 c/mM) labeled *E. coli* 15T⁻. The cells were stored in CO_2 and there was no observed latent-image fading. (Exposure: 1 day for 0τ and 4 days for 7τ and for unlabeled cells, development time: 1 min.). [Chi square test: 0τ, $P > 0.05$; 7τ, $P \ll 0.005$.]

TABLE V

THE SEPARATION OF THE OBSERVED GRAIN DISTRIBUTION INTO THREE POISSON DISTRIBUTIONS

Time	Fraction of cells with label	Fraction of labeled cells with small units	$\bar{g}_l$	$\bar{g}_l/\bar{g}_s$
3τ (Figs. 19, 23)	0.70	0.38	5.4	5.4
4τ (Fig. 20)	0.41	0.57	6.1	6.4
7τ (Figs. 21, 24)	0.046	0.48	5.0	4.6
7τ (Fig. 22)	0.048	0.23	5.8	3.2
		Average 0.4		5
2τ calculated from average	1.0	0.65		

data, is based on two relatively accurate numbers—the number of zero-grain cells and the graphically estimated number of labeled cells. The best average description we can give for 0τ cells is that approximately 1/2 of the cells contain 4 large units and 1/2 contain 4 large and 4 smaller units. We have not ruled out the possibility of a small number of cells with only 3 units (2). If 2 per cent of the 0τ cells had 3 units, we would expect 0.5 per cent unlabeled cells in the 2τ population. This fraction is near

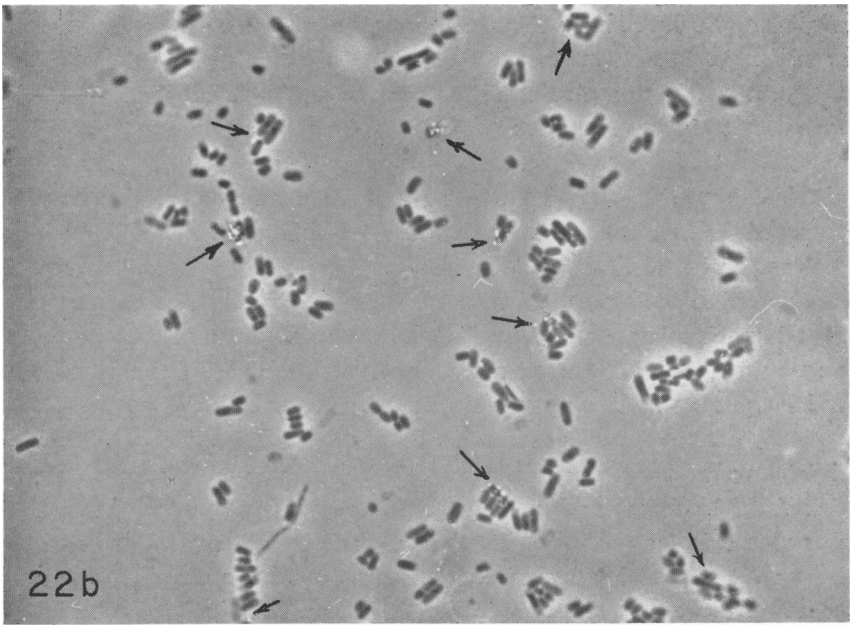

FIGURE 22*b* Photomicrographs of radioautographed 7τ cells. (Arrows indicate cells that have grains associated with them.)

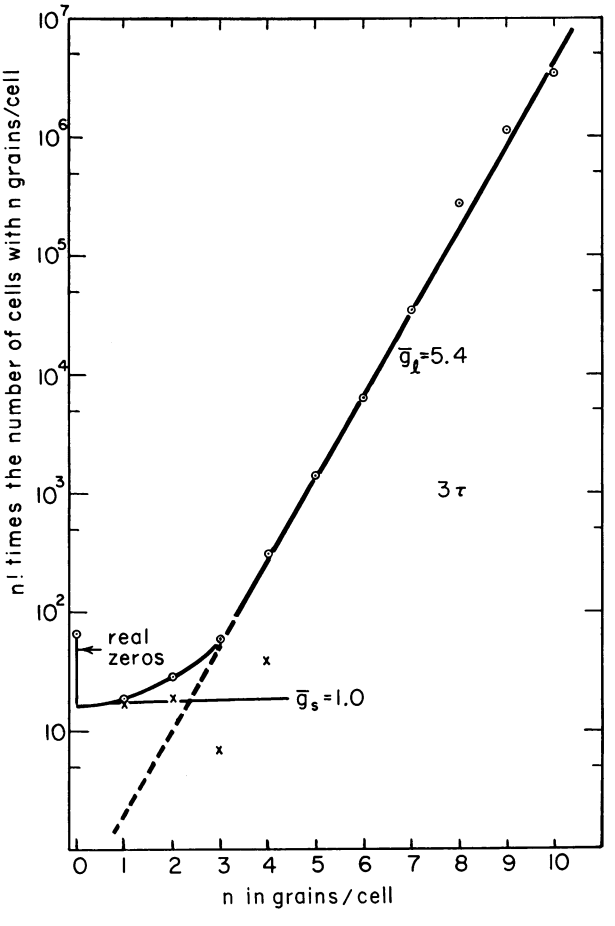

FIGURE 23 A plot of log [$n!$· (number of cells with n grains)] *versus* n for 3τ cells labeled with H^3T. The data are from Fig. 19. The observed distribution has been resolved into the sum of three distributions which have average values of gr/cell of $\bar{g}_l$ (large units), $\bar{g}_s$ (small units) and $\bar{g} = 0$ (cells with no labeled units).

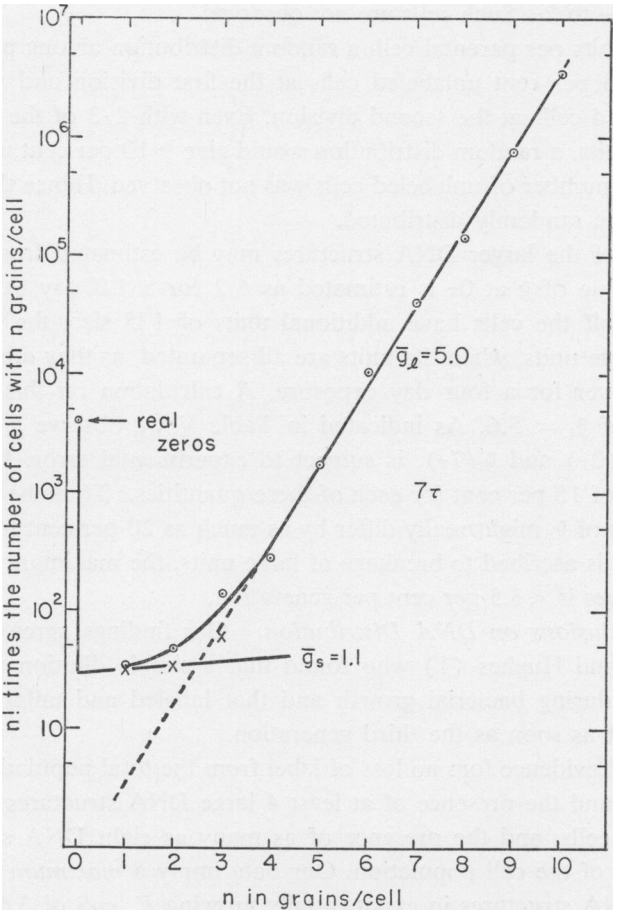

FIGURE 24 A plot of log [$n!$ · (number of cells with n grains)] *versus* n for the 7τ H^3T data of Fig. 21. The observed curve has been reduced into components as in Fig. 23.

the limit of detection by our methods. Our data are compatible with <4 per cent of the parental cells containing 3 units. The reasonable agreement between the 0τ data and a Poisson distribution (*cf.* Fig. 22) suggests that most of the cells have about the same DNA content. Differences of a factor of 2 between appreciable fractions of the population would go undetected but if >2 per cent of the cells had a very low DNA content compared to the average, we would find >1 per cent unlabeled cells at 0τ to 2τ. Such cells are not observed.

With only 4 units per parental cell, a random distribution among progeny would give $(1/2)^4$ or 6 per cent unlabeled cells at the first division and $(3/4)^4$ or 31 per cent unlabeled cells at the second division. Even with 2/3 of the parental cells labeled with 8 units, a random distribution would give >10 per cent unlabeled cells at 2τ. This large number of unlabeled cells was not observed. Hence the large DNA structures are non-randomly distributed.

The stability of the larger DNA structures may be estimated from the data of Fig. 22. The value of $\bar{g}$ at 0τ is estimated as 6.2 for a 1.0 day exposure. Since approximately half the cells have additional units of 1/5 size, the average grain count for the large units, when the units are all separated, as they are at 7τ, should be somewhat lower for a four day exposure. A calculation on this model yields for 7τ a value of $\bar{g}_l = 5.6$. As indicated in Table V we observe $\bar{g}_l = 5.8$. Each measurement, $\bar{g}(0\tau)$ and $\bar{g}_l(7\tau)$, is subject to experimental error. We estimate a maximum error of 15 per cent for each of these quantities.[5] Thus the predicted and calculated values of $\bar{g}_l$ might really differ by as much as 20 per cent for the 7τ data. If this difference is ascribed to breakage of large units, the maximum breakage rate for these structures is <3.5 per cent per generation.

F. *Conclusions on DNA Distribution.* Our findings agree with those of Painter, Forro, and Hughes (1) who found that a simple dilution of DNA label does not occur during bacterial growth and that labeled and unlabeled cells are observed at least as soon as the third generation.

We also found evidence for: no loss of label from the total population for several cell generations and the presence of at least 4 large DNA structures in at least 96 per cent of the cells, and the presence of as many as eight DNA structures in a sizeable fraction of the cell population. Our data imply a *maximum* breakage rate for the large DNA structures in exponentially growing *E. coli* of 3.5 per cent per

[5] The estimate is arrived at in the following way. The best data at 0τ and 7τ are shown in Fig. 22. The developing procedure used in this experiment gave a low background count and very small grains. Under such conditions the limit of linearity of the detecting system is $\bar{g} \sim 6$ grains/cell. At the edge of the preparation used to obtain the 0τ data a value of $\bar{g} = 5.2$ grains/cell was obtained compared to 6.2 obtained in the center. We take this difference of 15 per cent as the uncertainty in $\bar{g}(0\tau)$. Because the measured 7τ fields were chosen in the same manner as the 0τ fields we assume the same uncertainty in $\bar{g}_l(7\tau)$. The sum of the two 15 per cent uncertainties gives about a 20 per cent uncertainty.

generation. Half this breakage rate would be easily detected in more direct micromanipulation experiments. Hence our measurements in no way contradict Forro and Wertheimer's (2) previously published demonstrations of chromosomal breakage.

Taylor, Woods, and Hughes (43) showed that plant chromatids are bipartite. Each chromatid is transmitted in half units to the daughter cells, half chromatids separating non-randomly after DNA replication. Our measurements on bacteria also preclude random DNA structures and permit a functional two strandedness for the bacterial DNA (54). The bipartite nature of bacterial DNA of *E. coli* B has been demonstrated at the molecular level by Meselson and Stahl (55).

The equivalent statements for *E. coli* 15T⁻DNA structures, based on our grain count data, are:

1. Every cell contains at least 4 large DNA structures.[6]
2. The four large parental DNA structures segregate into the four daughter cells of the second generation.
3. These structures are conserved in succeeding generations. (Breakage is less than 3.5 per cent per generation.)

Our binary analysis, for cells growing in liquid medium has led to a description of DNA inheritance which is in essential agreement with that based on the micromanipulation experiments of Forro and Wertheimer (2).

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[6] Because ~ 90 per cent of the label is in four large structures, we are omitting the discussion of the behavior of the smaller DNA structures in the cells. We are also ignoring the possibility that 4 per cent (or less) of cells may have only three large DNA structures.

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