THE ORGANIZATION AND REPLICATION OF DEOXYRIBONUCLEIC ACID IN THYMINE-DEFICIENT STRAINS OF *ESCHERICHIA COLI*

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SUMMARY

1. An autoradiographic study of the distribution of incorporated [^{3}H]thymidine among the progeny of cells of the thymine-requiring strains of *Escherichia coli*, 15T$^-$ and B-3, has been made.

2. The distributions are markedly heterogeneous and are characterized by the presence of a small number of cells containing most of the label.

3. Experiments with *E. coli* 15T$^-$ exposed to label for less than one division time are interpreted as revealing the presence in this organism of two large DNA-containing structures which (a) must separate at cell division (b) may be perpetuated intact for many successive cell divisions, but (c) are subject at random to a finite probability of fragmentation during cell division. The possibility that these structures may be the two subunits of one larger structure is discussed.

INTRODUCTION

In a previous communication[1], it was reported that the distribution of incorporated [^{3}H]thymidine among the progeny of cells of a thymine-requiring mutant of *Escherichia coli* (strain 15T$^-$)[2], is very heterogeneous. We wish to discuss these results more fully in the context of some new findings.

MATERIALS AND METHODS

Bacteria

The bacterial strains used were the thymine-requiring organisms, *Escherichia coli* 15T$^-$, and *Escherichia coli* B-3, the latter initially isolated by Dr. S. BRENNER and obtained through the kindness of Dr. N. SYMONDS.

Media

The liquid culture medium was a phosphate-buffered, mineral salts medium known as M-9[3], supplemented with 4 μg/ml of either non-radioactive or [^{3}H]thymidine (Schwarz Laboratories Inc., Mt. Vernon, N. Y.). Specific activities ranging from 360 mC/m*M* to 3.0 C/m*M* were used during the course of the work.

Abbreviation: DNA, deoxyribonucleic acid.

Agar medium consisted of 0.8 % nutrient broth and 1 % Bacto-agar sometimes supplemented with varying amounts (40 or 400 μg/ml) of non-radioactive thymidine. In a few experiments, M-9 medium containing 1 % agar and varying quantities of thymidine was used.

Washing medium consisted of M-9 without glucose, supplemented with varying concentrations (4 or 400 μg/ml) of thymidine.

Labeling of cells

Radioactive liquid medium was inoculated by simple dilution of unlabeled log-phase cells growing at concentrations between 1 to $5 \cdot 10^8$/ml. The dilution factor was at least 1 to 10 in order not to alter appreciably the specific activity. No detectable change in the growth rate of the cells following inoculation occurred with this procedure. Cell counts were performed with a Petroff-Hausser counter. The extent of growth on label was an experimental variable to be discussed with the results. Radioactive cells were washed 2 or 3 times and resuspended in chilled washing medium.

Labeling was performed at 30° for *E. coli* 15T$^-$ and 37° for *E. coli* B-3.

Growth on agar and micromanipulation

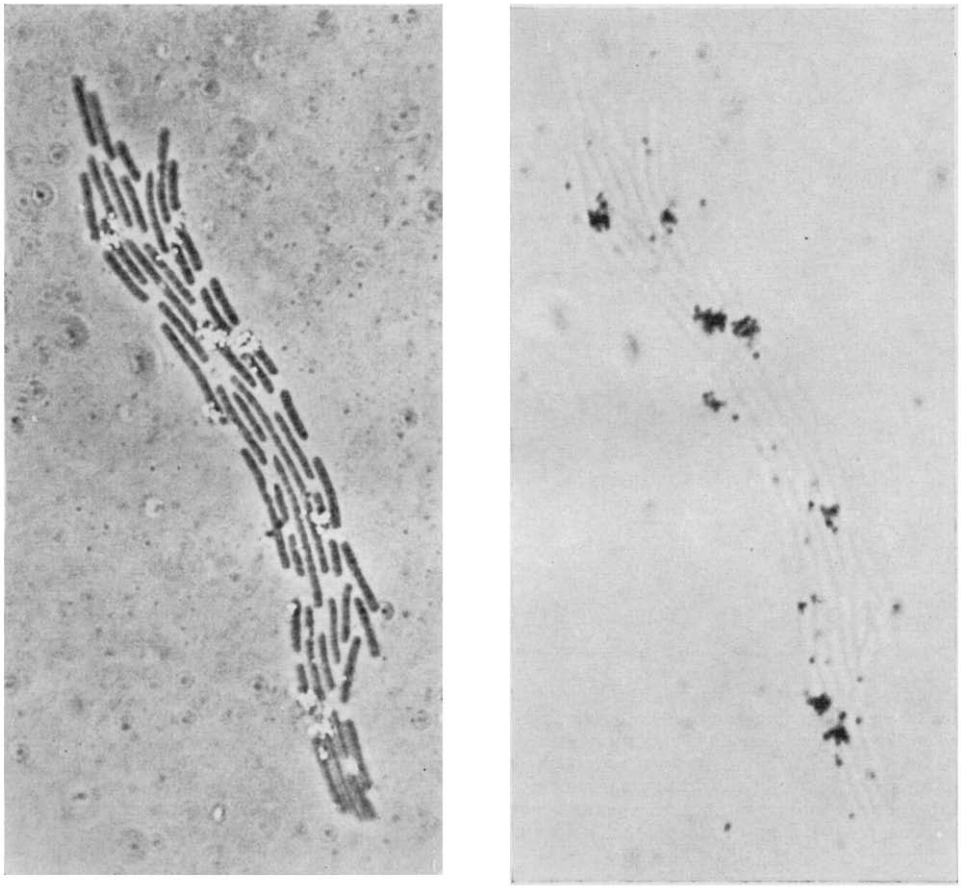
Small droplets of labeled cells were transferred to thin layers of non-radioactive agar medium deposited on 22 × 60 mm No. 2 coverslips. After cleaning, these coverslips had been pretreated for subsequent autoradiography by dipping into a solution of 0.1 % Knox deionized gelatin and 0.01 % chrome alum. Such coverslips are sufficiently thick (0.18–0.25 mm) to permit handling without significant breakage in autoradiography. The coverslips bearing the agar were placed on moist chambers. Two types of experiments were performed. On some coverslips, individual cells on the agar were permitted to grow to small colonies of varying size. On other coverslips, individual cells were isolated initially by micromanipulation and complete progeny lines isolated from these for several generations. The micromanipulation was performed by dragging cells on the agar surface with microneedles using a Chamber's micromanipulator. The position of individual cells was recorded by means of a graduated microscope stage with suitable reference markings on the coverslips. Usually, the incubation on agar was performed at room temperature (22 ± 2°) although some colonies were grown at 37°.

Autoradiography

The coverslips were handled for stripping-film autoradiography as previously described[4] except that the concentration of parlodian solution was reduced to 0.5 % to limit the absorption of β-rays by this layer. Cells isolated by micromanipulation were located by coordinate transformation on the graduated microscope stage of a Zeiss W-2 phase-microscope. Grain-counting was performed in brightfield under oil at 2000 × magnification. Recorded grain-counts were corrected for the trackiness that can be seen in such tritium autographs. It is not possible to count accurately more than a few disintegrations emanating from the same small locale (approx. 1 μ^2) because of the short-range of the low-energy tritium electrons. For grain-counts in excess of about 6 from such "point" sources of activity, the count will, in general, underestimate the activity compared with a distributed source containing the same amount of radioactivity.

RESULTS

When *E. coli* 15T⁻ or *E. coli* B-3 is grown for many generations in radioactive medium to label fully the DNA, the subsequent distribution of this incorporated label to the progeny of individual cells is illustrated by the colony shown in Fig. 1. More of the detail of such clonal distributions can be seen in Fig. 2, which shows the grain-count data of typical progeny-lines from micromanipulation experiments. Notable and typ-



a b

Fig. 1. Autoradiograph of colony of *E. coli* 15T⁻ grown from a single cell fully-labeled with [^{3}H]-thymidine. Note the clustering of grains over relatively few cells, indicating the heterogeneous distribution of the original DNA among the progeny. (a) Phase to show cells clearly. (b) Brightfield to show photographic grains clearly.

ical features of the distributions which have in part been summarized previously[1] are: (a) All-nothing distribution of label at division occurs frequently and can appear as early as the 3rd generation (8 progeny cells). (b) Such all-nothing distribution to daughters takes place successively in some sublines for several generations. In almost every progeny-line one can find at least one example of such perpetuated all-nothing

divisions. In some cases, these have been seen to occur for as many as 7 successive generations. This number might be even higher if progeny-lines were isolated for more generations. (c) In other sublines, in addition to all-nothing splits, one finds examples of partition of the label to both daughters. This partition leads to the appearance of different numbers of hot-spots in different clones in generations after the third. (d) Search for colonies or progeny-lines with all-nothing distribution at the second generation produced a few suggestive examples but only at low frequency. (e) Partition of the label at the first division was always approximately equal (with the exception of one progeny-line characterized by many morphological abnormalities).

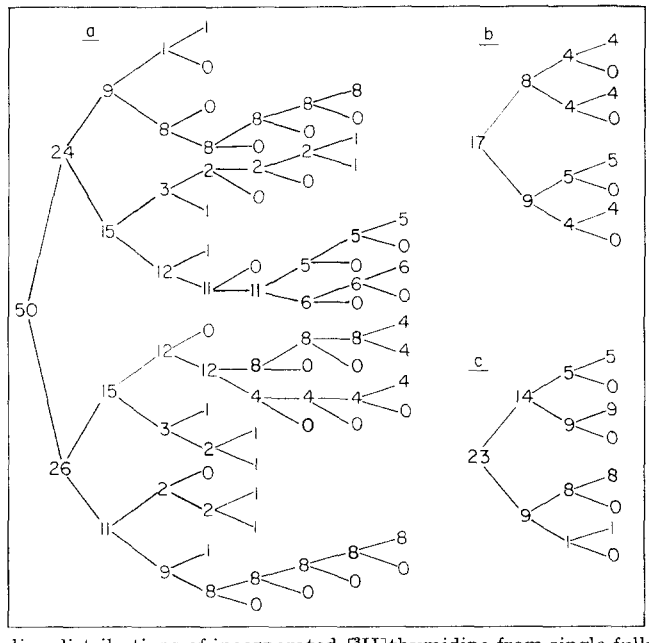


Fig. 2. Progeny-line distributions of incorporated [^{3}H]thymidine from single fully-labeled cells of *E. coli* 15T$^-$. *a* is a reconstruction from grain-counts collected from individual cells of the 7th or 8th generation and precise knowledge of the sibling relationships in the family tree by means of micromanipulation. Sublines terminating in 0 or 1 are not extended beyond their generation of origin. *b* and *c* are analogous reconstructions for progeny-lines whose grain-counts were made at the third generation. These clones show a remarkable incidence of all-nothing distribution of label among sibling-pairs at the third generation. The original cells for these two clones were siblings of a cell which completed division at the moment of isolation.

A simple interpretation of these experiments is that the individual fully labeled cells have a minimum of four DNA containing structures which must separate from each other at division and which are subject to a certain probability of fragmentation during cell division.

In order to explore further the significance of this number four, experiments were performed in which cells of *E. coli* 15T$^-$ were grown on label for one-half a generation time or less, preceding study of the distribution of label. Before presenting these results, it will be useful to discuss briefly the possible relations of DNA synthesis to the morphological features of the division cycle in *E. coli* 15T$^-$.

In Fig. 3a, we have represented schematically the growth of *E. coli* 15T$^-$ on M-9 medium. We have not specifically employed stains for nuclear bodies. However,

cells observed in phase-contrast or in increased-contrast brightfield after autoradiography appear to contain relatively transparent objects. From the shape, location, and number of these objects in relation to cell elongation in the division cycle, it seems reasonable that they can be identified as the so-called nuclear-bodies described by others[5]. Thus, we have drawn a cell which has just divided as containing one such "nuclear body".

It would be convenient for these kinds of experiments, if DNA synthesis were confined to a limited portion of the division cycle. This question has been examined in our laboratory[6], and by others[7] with the result that such a restricted interval of

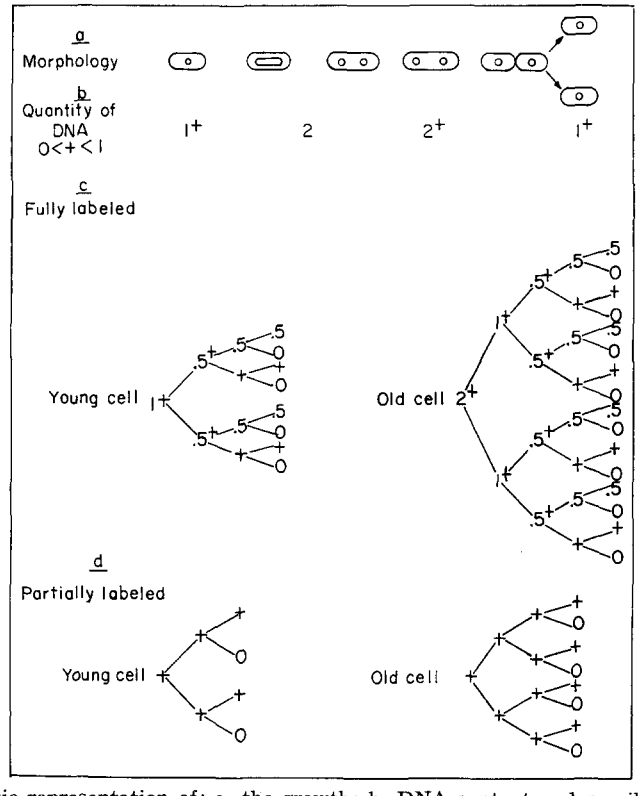


Fig. 3. Schematic representation of: a, the growth; b, DNA content and possible distributions of labeled DNA among progeny of c, fully-labeled cells and d, cells labeled for only part of a generation time.

synthesis does not seem to occur during logarithmic growth. This being the case, one cannot make the assumption that the completion of synthesis of any fundamental DNA-containing structures within a bacterium is necessarily coincident in time with the separation of two daughter cells. Rather, it seems more general to indicate the DNA-content of cells in the manner shown in Fig. 3b. Here, for sake of simplicity, we have made the assumption that there is one fundamental DNA-containing structure with a definite quantity of DNA, represented by the number 1. A cell which has just divided is depicted as having in addition to one copy of this fundamental unit a certain amount of DNA indicated by the + symbol, representing nucleic acid already synthesized in the duplication of this unit. Completion of this duplication is

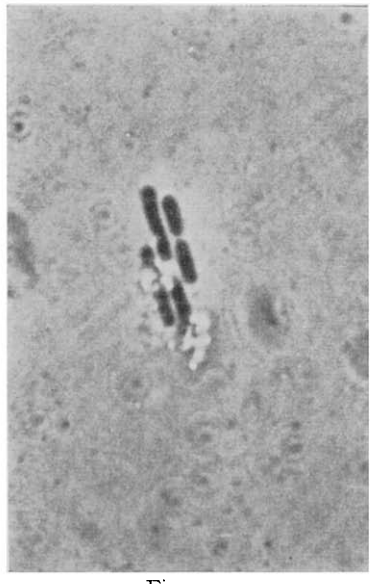


Fig. 4a.

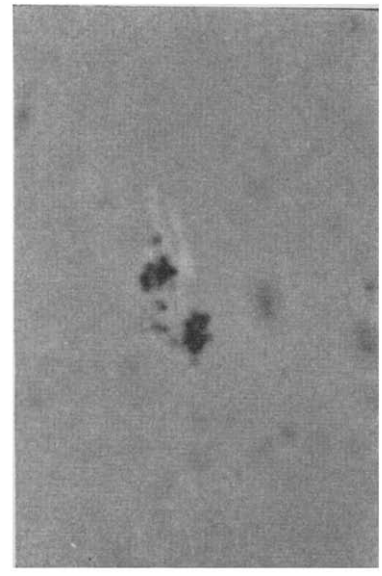


Fig. 4b.

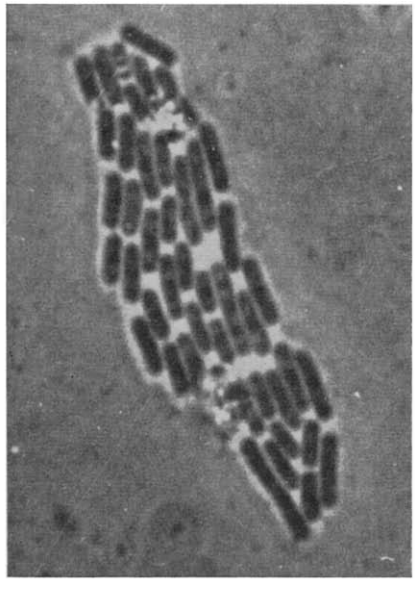


Fig. 5a.

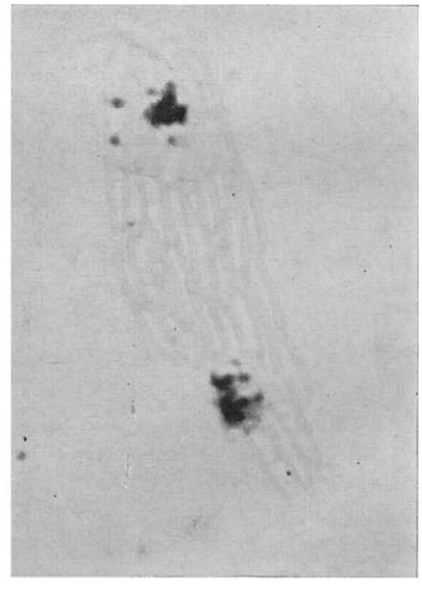


Fig. 5b.

symbolized by the number 2. Because of the continuous nature of DNA synthesis, each of these two units is visualized as proceeding immediately with its replication and concomitant DNA synthesis, symbolized by 2^+, prior to cell division.

In terms of this model, we have depicted the hypothetical results to be expected in a study of the progeny distribution of labeled DNA. The case of fully-labeled cells is contrasted with that of cells labeled for a short time, Fig. 3c and d. For these

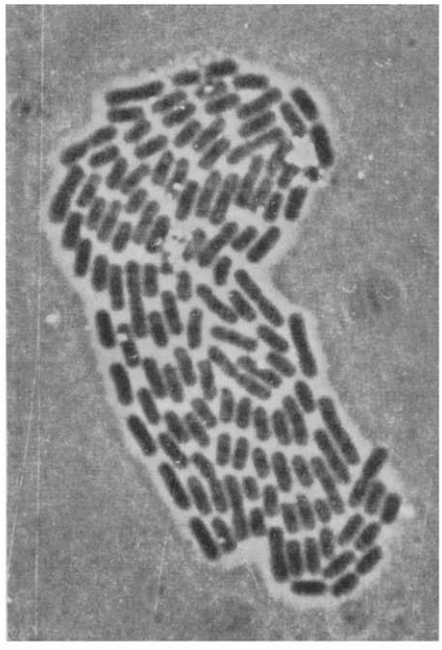


Fig. 6a.

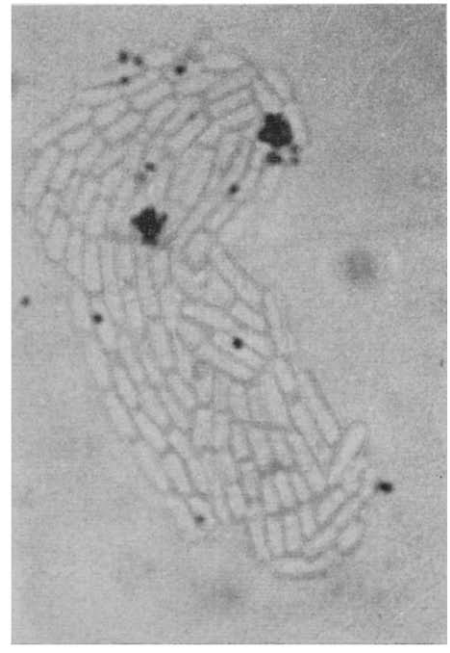


Fig. 6b.

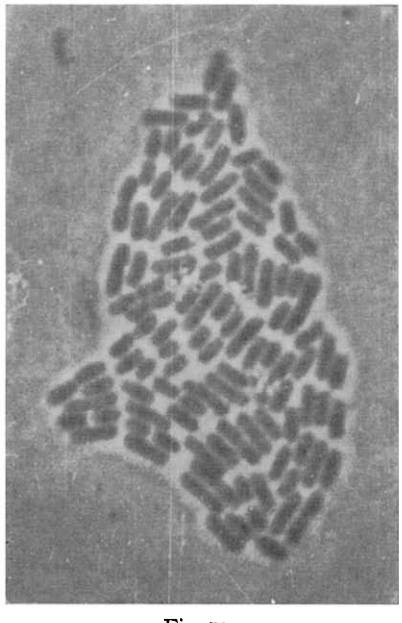


Fig. 7a.

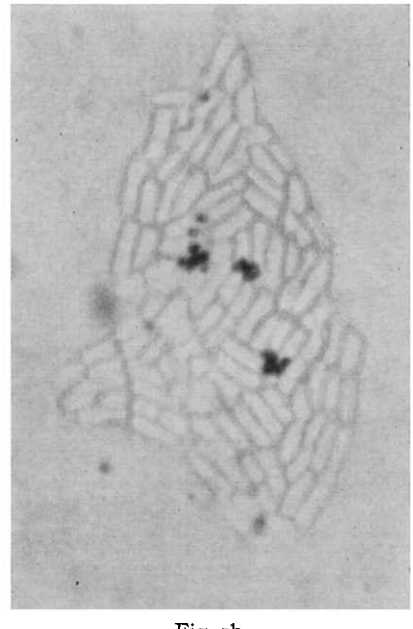


Fig. 7b.

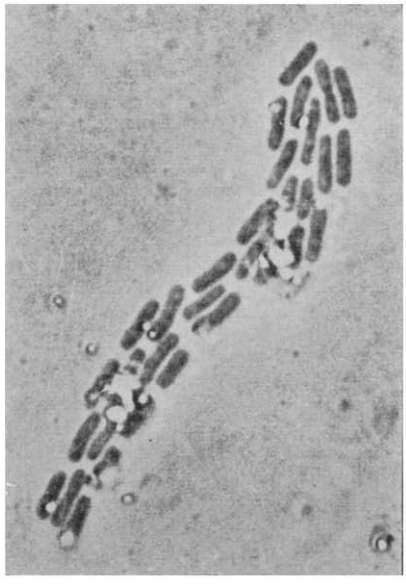


Fig. 8a.

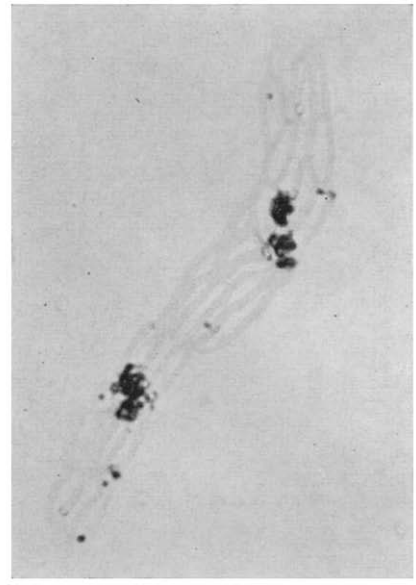


Fig. 8b.

Figs. 4–8. Autoradiographs of colonies of *E. coli* 15T⁻ grown from single cells labeled with [^{3}H]-thymidine for one-half of a generation-time or less. See text for description. a, phase; b, brightfield.

predictions, we have further assumed that the fundamental unit is bipartite and the two subunits are required to separate in replication. Incomplete subunits containing label are represented simply as + without regard to relative amounts of label.

Figs. 4, 5, 6, 7 and 8 are colonies representative of the types of distribution of label that can be found with exposure to radioactive thymidine for one-half of a generation time or less. Fig. 4 shows a 4 to 8-cell colony with two major clusters of activity representing first division separation of two labeled DNA-containing structures, followed by 1 to 2 generations of all-nothing segregation. Figs. 5 and 6 illustrate examples of 6th and 7th generation colonies respectively each revealing the major part of their tritium label in the form of two clusters of grains. Besides the hot-spots, such colonies contain some individual grains, the numbers of which have varied considerably in different parts of our experiments. We shall say more about these isolated grains later. In addition to colonies with two hot-spots, there occur others with 3, 4 and larger numbers of hot-spots. Fig. 7 is an example of one with three. Fig. 8 illustrates a colony with 4 such spots; these are grouped, however, in pairs over daughters at the fourth or fifth generation (one pair is not well resolved in the photograph).

When reconstructions from progeny-line experiments are made, the distributions are typical of those shown in Fig. 9, if one eliminates from consideration cells containing only one or two grains. With respect to hot-spots, the distributions follow the expectations depicted in our model for cells early in their division cycle with one additional feature noted previously for fully-labeled cells. There is some probability of sharing the radioactivity between daughters at divisions after the first. The model we have suggested would predict an overall simplification of the distributions by

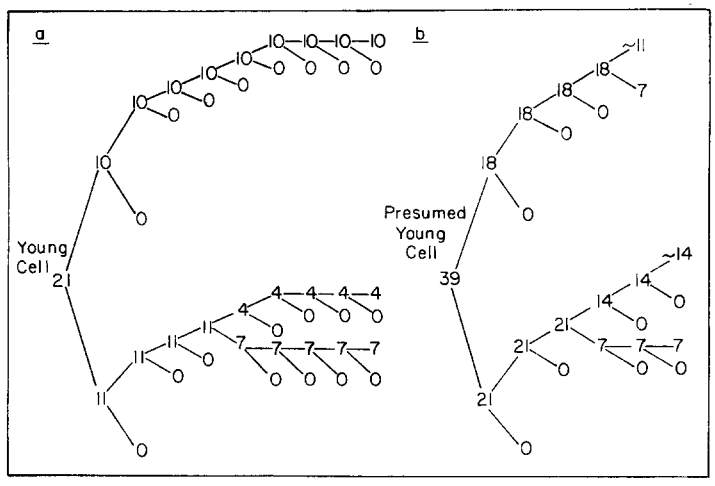


Fig. 9. Progeny-line distributions of hot-spots of incorporated [^{3}H]thymidine from single cells of *E. coli* 15T$^-$ labeled for less than 0.5 generations. a, reconstructed from micromanipulation data; b. probable reconstruction from colony of Fig. 8. Often the location of cells with respect to one another in small colonies permits reconstruction of sibling relationships for several generations.

limiting the time on label. This picture is sustained by the data given in Table I. This shows rather crude frequency distributions of the number of obvious individual hot-spots in randomly selected colonies derived from cells labeled for 5.5 and 0.5 generations respectively. These were approximately 7th or 8th generation colonies in each case. Since there was no selection of the initial cells giving rise to these colonies, the spread in numbers of hot-spots per colony reflect in part the spread in initial complexity attendant on the age distribution of the starting cells. Attention is called to the fact that in spite of this complexity approximately 10 % of the colonies labeled for 0.5 generations contain only two hot-spots.

The conclusions we feel can be drawn from these observations are the following. (a) In these thymine-requiring strains of *E. coli* there exist large DNA-containing structures which can remain intact for many successive replications. (b) The youngest cells of *E. coli* 15T$^-$ contain two of these structures. (c) These two structures have the further property that they must separate from each other at division. (d) These structures are subject to an undetermined probability of fragmentation during the cell division cycle.

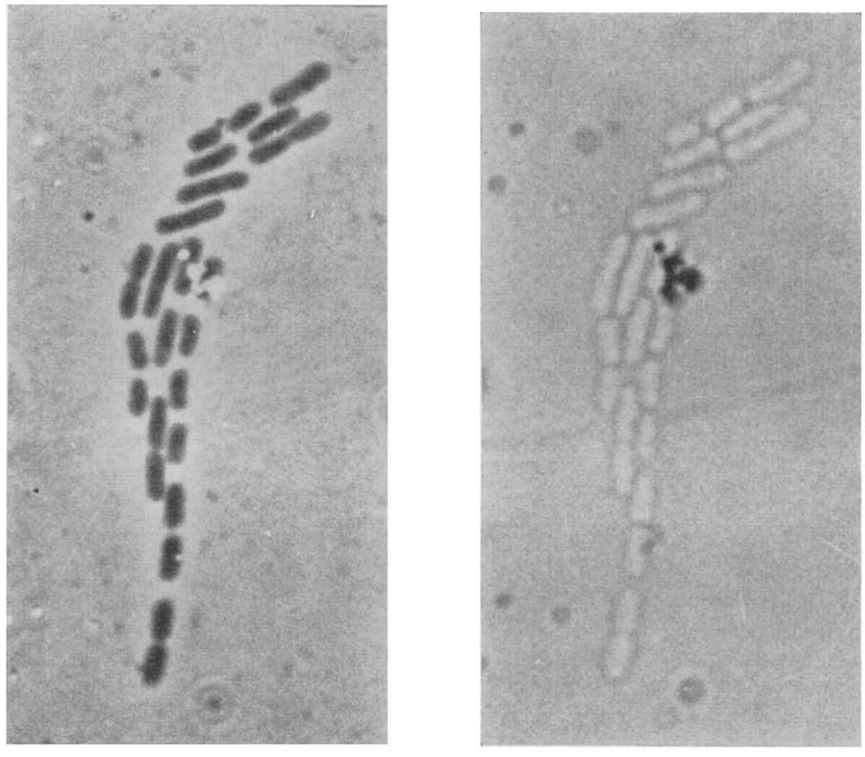
Regarding the last conclusion, we specifically consider the sharing of label by daughter cells (at divisions after the 1st for young cells labeled for less than one division, or after the 2nd for young fully-labeled cells) to result from some form of disruption in the continuity of the DNA-containing structure. The alternative that there exists in the initial hot cell more than two objects which are physically in-

TABLE I

DISTRIBUTION OF COLONIES WITH A GIVEN NUMBER OF HOT-SPOTS

Labeled for	*0*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*Total*
5.5 generations	0	0	0	2	12	17	19	16	11	4	6	4	2	2	3	98
0.5 generations	1	0	11	26	25	15	13	6	8	0	0	3	0	0	0	108

dependent and thus able to segregate randomly to daughters at division seems unlikely, since this would give a low probability of finding a colony with just two hot cells at the 7th generation. For example, if each of these hot-spots represented the random association of two independent objects for six successive divisions, the probability of finding such a clone would be $(0.5)^{12} = 1/4096$. As indicated previously, we find about 10 % of such colonies.



a b

Fig. 10. Autoradiograph of a colony of *E. coli* 15T⁻ grown from a single cell partially labeled with [^{3}H]thymidine showing only one cluster of grains. a, phase; b, brightfield.

A search was made among colonies from partially-labeled cells for any containing only one hot-spot. A few rare examples could be found (Fig. 10). A possible explanation of these in terms of our previous discussion of the relation of DNA synthesis to the division cycle can be ventured.

Occasionally cells may occur whose morphological division is more closely correlated with the completion of synthesis of DNA-containing substructures than is true for most cells. If the time of removal from label was coincident with cell division in such cases and the daughters of such a division became separated during the washing procedures, they could give rise to one-hot-spot clones. This possibility, plus the rarity of such clones, renders more extensive speculation on their significance inadvisable without further data. The occasional occurrence of second division all-nothing distribution in clones from fully-labeled cells are subject to analogous interpretation.

As noted previously, the colonies and progeny-lines from partially labeled cells show a variable number of isolated grains in their autoradiographs. In our first experiments with fully-labeled cells, the percentage of the total activity represented by this non-clustered activity was small enough (around 25 %) that it was construed simply as part of the general pattern of distribution. In some of the partial labeling experiments, however, as much as 80 % of the grains appeared in non-clustered form. Whether this unexpected finding reflects an uncontrolled technical difficulty or represents something of biological interest is not yet clear. Since hot-spots have been a common feature of all our experiments and since we have examples of partial labeling experiments in which almost all the activity is clustered, we have felt justified in discussing the partial labeling experiments with consideration of the hot-spots only. Some of the distributed activity undoubtedly arises from asymmetric fragmentation of the DNA-structures. However, the variability in amount of this

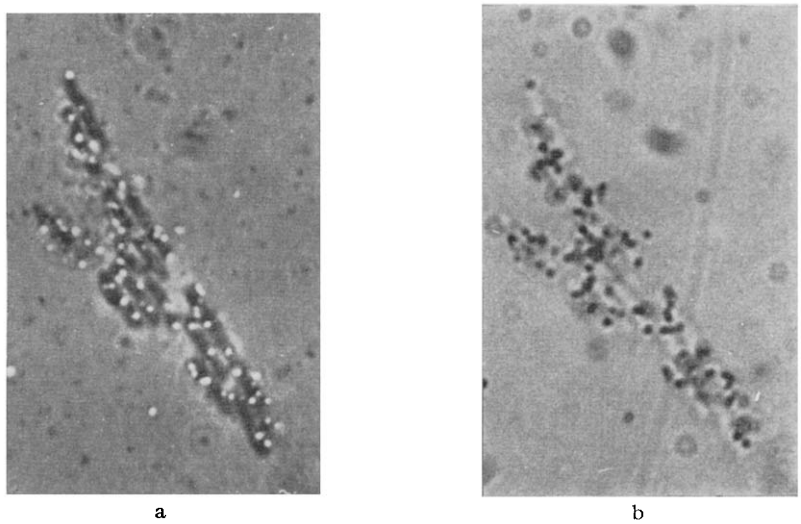


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Fig. 11. Autoradiograph of a colony of a uracil-requiring *E. coli* strain grown from a single cell fully-labeled with [^{3}H]uridine. a, phase; b, brightfield.

activity confuses attempts to estimate the fragmentation frequency per division.

In the schematic diagram of Fig. 3, depicting the morphology on M-9 medium, we have shown a young cell as possessing one nuclear body. It should be pointed out that on agar containing nutrient broth, cells of *E. coli* 15T$^-$ grow longer than on M-9 and young cells usually contain at least two nuclear bodies. This accounts for the longer cells seen in the photographs.

No easily detectable changes in the essential features of the hot-spot distribution of label have been found to occur over the 9-fold range of specific activity of thymidine used. The results also have seemed relatively invariant to changing the nutrient of the agar medium to M-9, the presence of different quantities of cold thymidine in the agar medium and the temperature of growth on agar. Fig. 11 shows a colony derived from a cell fully-labeled with [^{3}H]uridine. The more random distribution without readily detectable hot-spots is in strong contrast to the thymidine experiments.

DISCUSSION

The young small cell of *E. coli* 15T⁻ grown on simple mineral medium seems to synthesize DNA as if this substance were organized into two large structures. Whether these two units are initially part of one larger unit which replicates in a semi-conservative[8] fashion or whether each represents the conservative replication of two separate structures cannot be decided from the experiments. The light-microscope appearance of one nuclear-body and the electron microscope picture[9] of one continuous structure can at best be considered weak evidence for the first interpretation. MESELSON AND STAHL[10] have reported experiments which indicate that the individual molecules of *E. coli* B replicate as if they consisted of two subunits. The hot-spots in our experiments indicate the presence of structures which are the order of 100 times larger than the molecules observed in the centrifuge experiments. If the results of the density-gradient experiments should prove true also of *E. coli* 15T⁻, it would be difficult to escape the conclusion that there exists in these organisms one large DNA-containing structure which replicates as if it were composed of two subunits, this form of replication in turn reflecting the semi-conservative form of replication of the individual molecules. With this interpretation, the behavior of these bacterial structures would be quite analogous in their mode of replication to plant chromosomes as shown by TAYLOR *et al.*[11,12]. One wonders if the fragmentation we see might also be the counterpart of sister-strand crossing-over seen in the chromosomal studies.

The notion of the presence in bacteria of structures analogous to chromosomes of higher organisms is not new. It has received particular support in recent times from the studies of bacterial recombination[13]. The amount of the material and the manner of transfer of genetic characters from the donor to the recipient cell would seem to imply the organization of many DNA molecules into a larger linear superstructure[14]. The nature of the material which binds the DNA together is not known. The experiments both on chromosomes and on bacteria could be explained by the presence of DNA as one large supermolecule replicating in the manner suggested by WATSON AND CRICK[15]. The comparative ease of obtaining DNA preparations relatively homogeneous in molecular weight from *E. coli* as seen in the experiments of MESELSON AND STAHL[10] would imply rather regularly spaced points of structural weakness in such a super-molecule. It seems more attractive to think that some other material serves to bind similarly-sized DNA molecules together. Whatever the nature of this linkage, it must allow the semi-conservative replication of individual molecules to be reflected in the mode of replication of the superstructure. Some models suggesting how this might occur have been discussed previously[11,16]. Reports in the literature[17–19] of the isolation of DNA-containing structures from bacteria lend hope for the characterization of the superstructure(s).

The fragmentation we have observed could conceivably occur within DNA molecules, in the material that may link them, or at the junctions between the two. If the first were true, and breakage of one molecule were equivalent to the observed event, the frequency of fragmentation is low enough and the size of the large structures is large enough that at most 1–2 % of the cell's DNA molecules would be broken in one division cycle. It is doubtful if these would have been detected in the gradient-density experiments of MESELSON AND STAHL.

Studies on the organization and mode of replication of the DNA of virulent

bacteriophage have presented somewhat conflicting results[20]. More recent experiments indicate that some of the DNA molecules of these viruses may be linked in a manner formally similar to bacteria.

The possibility that the radiation associated with tritium-decay during the experiment influences the results cannot be evaluated unequivocally at present. Artificial aggregation of DNA by the radiation would seem unlikely in view of the range of specific activities employed, unless the dose-response curve for such a phenomenon were quite peculiar. Progeny-line experiments show that any such hypothetical aggregation is at least compatible with normal growth patterns. The fragmentation might result from the radiation, but again the lack of obvious gross changes in the distribution patterns at different specific activities does not favor this idea.

The generality of these results for non-mutants and other species of bacteria needs exploration. It is not inconceivable that under different circumstances, the bacterial chromosomes will prove to be more ephemeral.

ACKNOWLEDGEMENTS

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