LOCALIZATION OF MACROMOLECULES
IN *ESCHERICHIA COLI*

I. DNA and Proteins

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ABSTRACT

If thin sections of *Escherichia coli*, labeled uniformly with tritium, are radioautographed calculations, based on the distribution of section sizes show that the number of H^3 decays per section should be very close to a Poisson distribution. We might, therefore, expect that the distribution of radioautographic grain counts among random cross-sections should follow a Poisson distribution. It can then be inferred that a deviation from a Poisson indicates a high concentration of label in a preferred region. This region can then be identified by analysis of serial section and comparison with electron micrographs. Sections of cells labeled with leucine-H^3 gave a Poisson distribution of grain counts, and it was concluded that proteins were distributed fairly uniformly throughout the cell. The situation was not changed if labeled cells were placed in chloramphenicol or if very short pulses of label were used. When *Escherichia coli* is grown in presence of chloramphenicol a major morphological change concerns the nuclear region: it becomes more regular in outline, nearly spherical, and occupies a smaller proportion of the cell length. The previously described association between DNA labeled with thymidine-H^3 and the nuclear region was confirmed by showing that the distribution of the label in the cell followed exactly the morphological changes of the nuclear region. It was also shown that the concentration of DNA in the nuclear region was at least 45 times higher than that of the cytoplasm. Several morphological features of cells grown in chloramphenicol and examined in the electron microscope are discussed.

The limit of resolution of radioautography, using tritium as a label, is of the order of 1 to 1.5 μ. In a previous note (5) we introduced a method by which this limit could be overcome, to some extent, in localization studies on microorganisms. The techniques used involved the statistical analysis of radioautographic grain counts over random cross-sections of thinly sectioned bacteria and the consideration of serial sections of individual bacteria. The localization of the label was correlated to the morphological structures seen in the electron microscope. We thus found evidence of a relation between the localization of deoxyribonucleic acid and the nuclear region seen in electron micrographs of *Escherichia coli*.

In this paper we shall use these methods of analysis to study the localization of incorporated leucine-H^3 and that of very short pulses of leucine-H^3. We shall also confirm the results obtained for DNA by showing that an experimental modification of the nuclear region, such as that brought about by growth in the presence of chloramphenicol, results in a parallel change in the distribution of incorporated thymidine-H^3. A brief description of electron microscopic observations on cells treated with chloramphenicol will be included.

The localization of RNA and of its possible sites of synthesis will be treated separately in a following article (6).

MATERIALS AND METHODS

Growth and Labeling: The organism used was *E. coli* T⁻U⁻, a thymine- and uracil-requiring mutant (2), obtained through the courtesy of Dr. S. S. Cohen. Cultures of *E. coli* 15 T⁻U⁻ were started from slants and grown at 24°C. without aeration in tubes containing 1 ml. of M-9 synthetic medium (1) with 4 μg./ml. of thymidine and 25 μg./ml. of uridine. Parallel transfers to control tubes lacking either one of the required compounds were made routinely to check for back-mutations. The cultures were maintained by serial transfers for several days before each experiment and were never allowed to exceed a concentration higher than 10^9 cells/ml. Logarithmic growth was maintained up to that concentration as shown by Petroff-Hausser counts and incorporation of leucine-H³ and thymidine-H³.

Under these conditions of growth, it was found that the cells presented a great uniformity with respect to cell length, growth rate, motility, and morphology. The generation time was approximately 140 minutes, giving a conveniently low rate of synthesis for pulse experiments.

Cells were labeled either by substituting 50 μc./ml. of thymidine-H³ (from Schwarz Labs., Inc.) with a specific activity of 3000 mc./mM, for the thymidine in the medium, or by adding 50 μc./ml. of DL-leucine-4,5,H³ (from New England Nuclear Corp.) with a specific activity of 3750 mc./mM, and growing the cells in this modified medium for 6 to 7 generations.

Short pulse labeling with leucine-H³ was achieved by adding 100 μc./ml. of leucine-H³ to cells growing exponentially. After the desired length of time the uptake of label was stopped by adding formaldehyde to a final concentration of 4 per cent. It was determined by radioautography and Geiger counting in a flow counter that addition of formalin stopped the uptake of leucine almost instantaneously.

Roberts *et al.* (25) have shown that in *E. coli* exogenous leucine appeared almost entirely as protein leucine. Van Tubergen (28) showed that in the case of DL-leucine-H³ only the L-leucine was utilized by *E. coli* 15 T⁻U⁻. In our preparations digestion with trypsin or chymotrypsin after formalin fixation of the labeled cells removed 70 to 75 per cent of the label. This proportion remained the same in the case of short exposures to the label (42 and 60 seconds). In all cases treatment with DNase or RNase did not remove a measurable amount of label.

The use of a thymidine mutant insured a high labeling of DNA in the presence of thymidine-H³.

Treatment with DNase removed 97 per cent of the label from the fixed cells, while RNase removed less than 5 per cent.

To achieve an experimental modification of the nuclear region, cells fully labeled with thymidine-H³ or leucine-H³ were grown in the presence of 50 μg./ml. of chloromycetin (from Parke Davis) for 60 to 90 minutes.

Fixation and Embedding: Cultures containing 2 to 4 × 10^8 cells were first fixed by adding formaldehyde, to a concentration of 4 per cent. After 3 hours the cells were precipitated by centrifugation and resuspended in 50 μl. of medium. Approximately 100 μl. of 2 per cent agar melted and brought to 45°C. were mixed with the cells and allowed to gel for a few minutes in an icebox. Fixation was then continued for 12 hours in the cold (4°C.) in a solution of 1 per cent OsO_4 and 0.4 M sucrose in 0.2 M acetate-veronal buffer at pH 7.0. The blocks were cut into small cubic pieces of about 8 mm.³ which were washed briefly in water, dehydrated through graded alcohols, and embedded in a mixture of 1:10 methyl methacrylate-*n*-butyl methacrylate, following standard procedures (11). Measurement in a gas flow Geiger counter of the amount of radioactivity lost to the various solutions revealed that only about 2 per cent of the thymidine-H³ and 4 per cent of the leucine-H³ were lost during the entire procedure. In both cases most of the loss occurred during the fixation and it is felt that it probably represents low molecular weight compounds.

Electron Microscopy: Thin sections (∼500 A thick) were cut with a Porter-Blum microtome and placed on carbon-coated grids. They were stained by floating the grids for a few minutes on a 1 per cent solution of lanthanum nitrate (14) or on a solution of lead hydroxide (30). The sections were examined and photographed in an RCA EMU-3B electron microscope.

Radioautography: Thicker sections, 0.2 μ thick, were cut in a similar manner and placed on a slide previously coated with a mixture of 0.5 per cent gelatin and 0.05 per cent chrome alum. The methacrylate was removed with amyl acetate. Random fields were photographed in a phase contrast microscope. The slides were then covered with a strip of Kodak Ltd. autoradiographic emulsion AR-10 (10) and exposed for suitable lengths of time. After photographic processing they were mounted in 65 per cent glycerin. The previously photographed fields were identified and the number of photographic grains associated with each cross-section of a cell counted, using phase contrast for identifying the sections and bright field for counting, and correcting for tracks whenever it seemed necessary. The practice of photographing random fields before placing the emulsion on the slide insured against bias in the selection of the sections to be counted and helped in identifying the

smaller sections, the presence of the emulsion bringing about a serious decrease of contrast. Longitudinal and oblique sections were also counted and used for qualitative interpretation, but not included in the statistical analysis. It was verified that no section of individual bacteria was lost during the procedure.

For serial sections a similar method was used. Successive sections of each cell in a given field were identified in photographs. Only cells oriented perpendicularly to the plane of the sections were selected for analysis. Another criterion was that the first section (at the tip of the cell) be not a grazing section. Such cells were followed through four or five sections. Averaging the number of grain counts over thirty to fifty cells gave an estimate of the longitudinal distribution of label. Qualitative consideration of longitudinal sections gave an estimate of the transversal distribution.

Since it is difficult to have an exact measurement of the size and shape of an average cell, theoretical distributions of label in serial sections were constructed as follows: each preparation for which such a model was to be made was photographed in the electron microscope and about 200 cross-sections of cells were measured and arranged by classes according to diameter. The probability of each class was taken to represent the proportional length occupied by that class in the cell (making the assumption that all cells have uniform diameters). A block diagram was constructed where the heights represented the volume of the region in which we assume the label to be contained for each position along the length of the cell. We then cut imaginary serial sections through this diagram and tabulated the area included in each section. The final result of such a construction is shown in Fig. 12(A). It accounts not only for the morphological characteristics of a given preparation, but also for the fact that most of the sections considered will not be perfectly perpendicular to the axis of the cell. It can be guessed that in this particular preparation the cells were not perfectly cylindrical. This was confirmed to some extent by examination of longitudinal sections.

RESULTS

Electron Microscopy: Figs. 1 and 2 show a comparison between a cell of *E. coli* 15 T⁻U⁻ grown under normal conditions and one from a culture which had been growing in the presence of chloramphenicol for 60 minutes. The morphology of the normal cells is in all respects similar to that of *E. coli* 15 T⁻, grown under similar conditions, previously described (5) and does not differ significantly from that of other strains of *E. coli* described by Kellenberger and his group (15, 16, 27) and various other authors (3, 18). Large

granules (approximately 600 A in diameter) stained by La(NO₃)₃ (5) and located preferentially at the poles of the cell appear also after lead hydroxide staining (Fig. 1). When the cells are stained with uranyl nitrate these granules are replaced by regions of low density of similar size and shape. They might be similar to the holes ("trous") described by Ryter and Kellenberger (27) in other strains of *E. coli*.[1]

When the cells are grown from 15 to 90 minutes in chloramphenicol the cytoplasm, cell wall, and 600 A granules show no obvious change. The most striking differences concern the nuclear region. It becomes shorter, occupying only from 45 to 55 per cent of the length of the cell (*versus* 67 per cent in normal cells). Its outline becomes smoother and more regular (Fig. 2). It seems, therefore, that the total area of contact between the cytoplasm and the nuclear area has somewhat decreased. The distribution of fibrillar material in the nuclear region appears unchanged, although the concentration seems to increase with time. As in normal cells, direct contact between these fine strands and the granular cytoplasm appears extensive at the interface between the two regions. Small islands of denser material appear often in the center of the nuclear region (Fig. 2). In the type of preparation illustrated in Fig. 2 the finely stranded material of the nuclear region seems aggregated into coarser filaments. Such filaments often show continuity between the cytoplasm proper and the central islands. If the cells are washed in uranyl acetate after fixation, as suggested by Ryter and Kellenberger (27), the filaments are much finer (Figs. 5 to 7) and such relations are not easily seen, although they might still exist.

The observations presented here are typical of cells grown in chloramphenicol and agree in general with the results of Kellenberger, Ryter, and Séchaud (15) on *E. coli* B treated with chloramphenicol and of Kellenberger and Ryter on *E. coli* K 12 treated with aureomycin (16). Some variations regarding the existence and size of "cytoplasmic islands" in the nucleus and the size and shape of the nuclear region itself do occur, according to growth rate, medium, cell density, and other variables. Accordingly, careful

[1] A recent paper by B. Cedergren and T. Holme (*J. Ultrastruct. Research*, 1959, 3, 70) has established a correlation between these "holes" and the presence of glycogen in the cell.
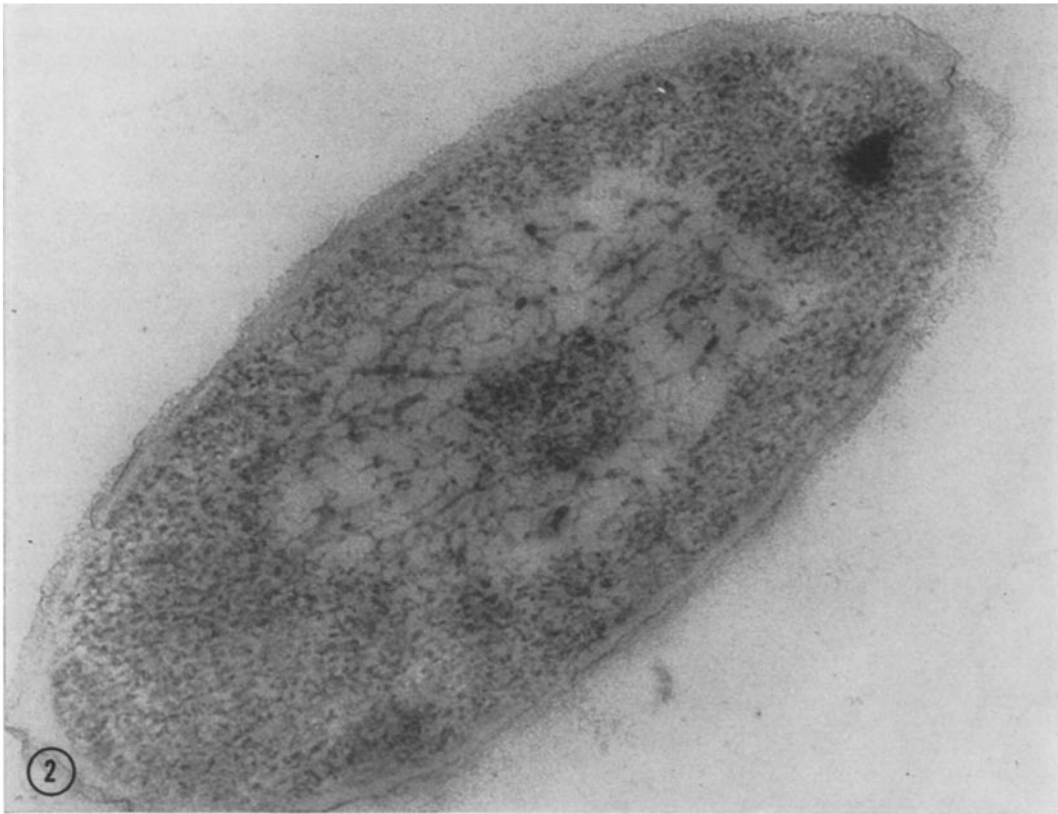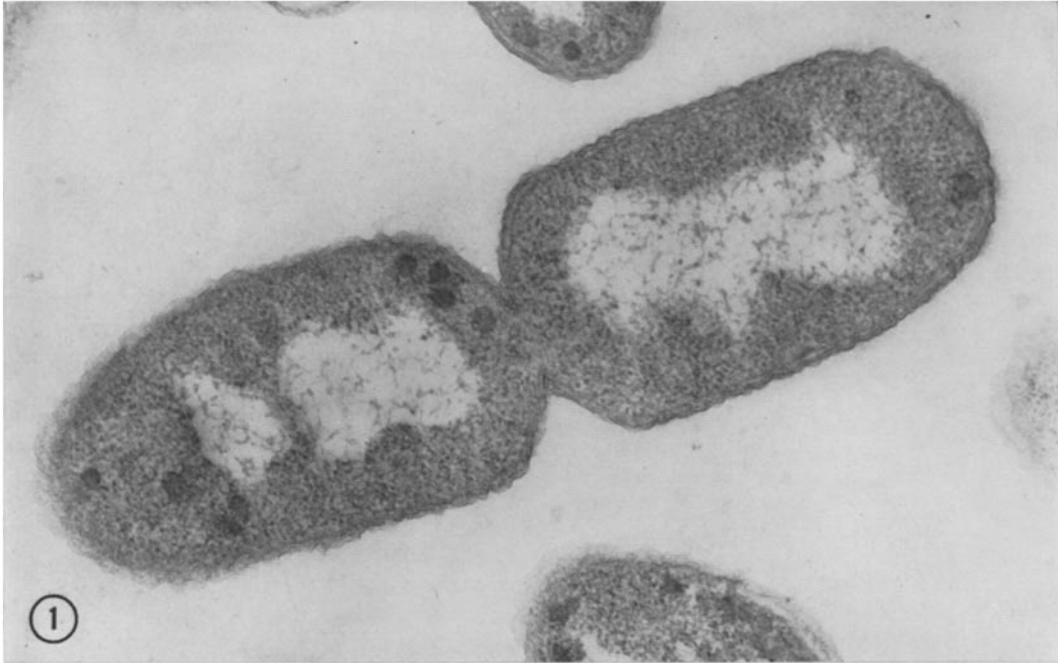
electron microscopic measurements were taken on each of the preparations used for radioautography.

In one preparation of cells grown for 90 minutes in chloramphenicol the occasional appearance of membranous structures, both in the cytoplasm (Fig. 8) and in the nuclear region (Figs. 5 to 7), was observed. These structures showed a variety of forms, appearing usually as a vesicle bounded by a single (Fig. 5) or a double membrane (Figs. 6 to 8). A continuity between the outer membrane and the plasma membrane was sometimes observed (Fig. 8). Such membranous structures have not been seen in any of numerous other preparations of normal or chloramphenicol-treated cells of various strains of *E. coli*. Neither have they been described, to our knowledge, by other authors. It, therefore, seems difficult to judge whether they are due to the action of chloramphenicol, whether they represent a normal feature of a certain stage of growth, or whether they are due to some other cause. Their presence does not seem likely to affect the radioautography results.

Radioautography. Analysis of Grain Counts from Randomly Selected Cross-Sections: If, in a given field, there were a number of cross-sections of cells of equal diameter, and if these cells contained a randomly distributed label, the number of grains associated with each section might be expected to follow the rules of a Poisson distribution:

$$P_i = \frac{a^i e^{-a}}{i!}$$

where P_i = probability that a section have i grains, and a = average number of grains per section. If we consider n classes of sections containing various amounts of label, the total probability that a section will have i grains becomes the sum of several Poisson distributions:

$$P_i = \sum_f^n P_f \cdot \frac{a_f^i e^{-a_f}}{i!}$$

where P_f = probability that a section belong to the fth class and a_f = average number of grains for fth class.

The quantity P_f represents, therefore, a distribution factor for the label in the cell. If the differences between a_f for various classes are large, for example if some sections include a labeled structure and others do not, then the distribution will differ greatly from a Poisson. Using the above formula it is possible, for a given average grain count, to calculate model distributions based on various hypotheses. We can then compare with the actual distribution and see which hypothesis best fits the data. The data necessary to calculate P_f and a_f are obtained by examination and measurements on a large number of electron micrographs from the same specimens as those used for radioautography. The results obtained from some such theoretical distributions are worth noting here. Three main classes of label distributions can be expected to give a grain distribution which is experimentally indistinguishable from a Poisson curve: (*a*) label randomly distributed in cytoplasm, (*b*) label randomly distributed in

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FIGURE 1

A dividing cell of *E. coli* 15 T⁻U⁻, stained with lead hydroxide. The nuclear region extends to about ⅔rds the length of the cell. Fairly deep projections of cytoplasm into the nuclear region and of nuclear region into cytoplasm can be observed. The nuclear material is slightly clumped. The cytoplasm has the usual granular appearance. Many areas of contact between the nuclear threads and the cytoplasmic granules can be seen. The large (550 to 600 A) stained granules are preferentially located at the pole (or what soon will become a pole) of the cell. The cytoplasmic membrane is visible in places. X 60,000.

FIGURE 2

A cell of *E. coli* 15 T⁻U⁻. Treated with chloramphenicol for 60 minutes, stained with lead hydroxide. The nuclear region is shorter, more nearly spherical, more uniform in outline. It occupies only ½ the length of the cell. The coarser clumping of nuclear material is due to small differences in the fixation. Notice the island of cytoplasmic-like material in the center. Many of the clumped nuclear fibers seem to link the island to the cytoplasm. Only one stained large granule can be seen. This is due to the growth condition, not to the effect of chloramphenicol. X 96,000.

FIGURES 3 and 4

Phase contrast micrographs of radioautographs from two consecutive serial sections of thymidine-H^3–labeled cells. The clumps of photographic grains appear as bright spots; the sections of bacteria are dark. There are many examples of sections unlabeled in one field and heavily labeled in the following field (arrows). At a the arrow points to a longitudinal section showing a similar effect. From their position in the cell, the labeled sections are judged to correspond to sections including nuclear material. The radio-autograph exposure was such that the average number of grain counts over the exposed sections is about 20. $\times$ 2300.

FIGURES 5 to 8

E. coli 15 T^-U^-, treated with chloramphenicol for 90 minutes, washed in uranyl acetate after fixation (27). These micrographs illustrate the appearance of membranous structures in this preparation.

FIGURE 5

A nuclear structure, limited by a single membrane, 40 to 50 A thick. An overlapping of the membrane seems to occur in the upper portion. The structure excludes nuclear material, but many nuclear fibrils seem to be attached to the membrane, or in contact with it. $\times$ 94,000.

FIGURE 6

A nuclear structure limited by a double membrane. The inside seems filled with a dense, homogeneous material. Again there is a close relation between the membrane and the nuclear fibrils. $\times$ 90,000.

FIGURE 7

A structure similar to that in Fig. 6, but smaller. $\times$ 87,000.

FIGURE 8

A cytoplasmic vacuole bounded by a double membrane. The outside membrane seems to be continuous with the cytoplasmic membrane. The separation of the cell wall from the cell is a fixation or embedding artifact. $\times$ 110,000.
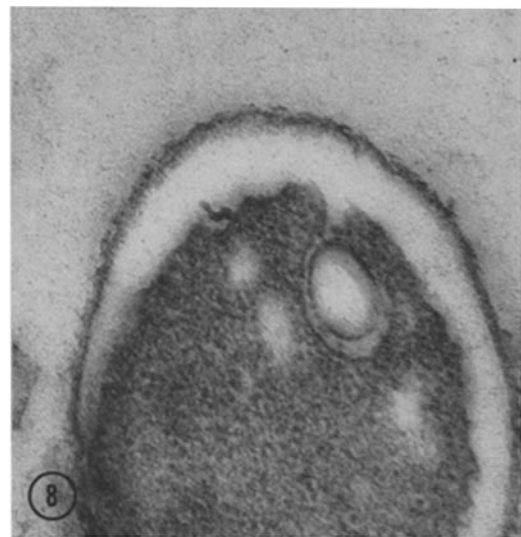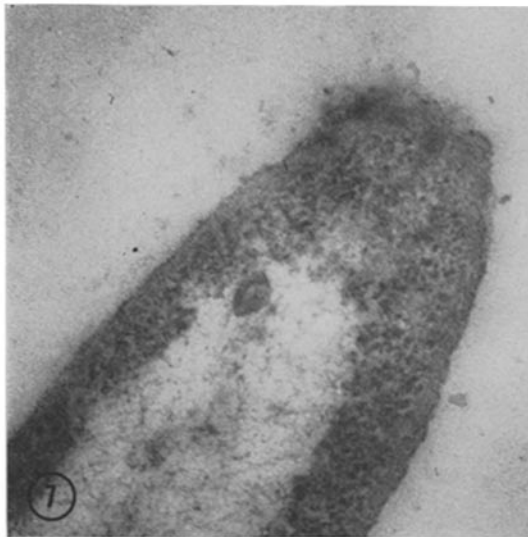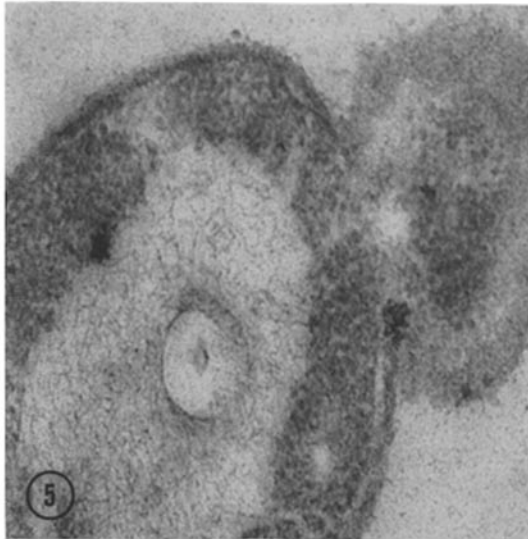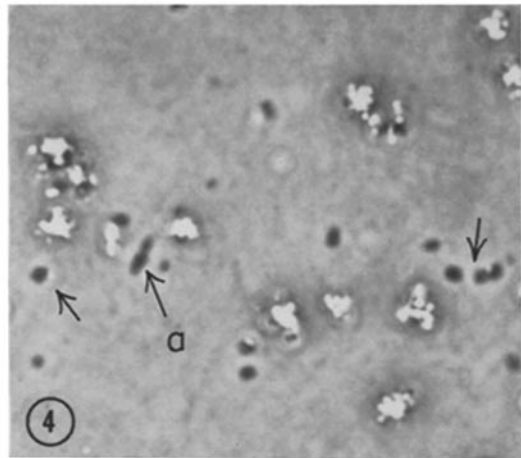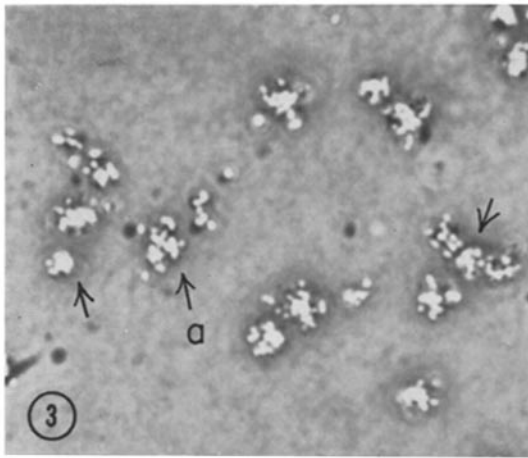
cytoplasm and nuclear region, and (c) label distributed in a thin layer on the periphery of the cell (e.g., cell wall or cell membrane). It is impossible to distinguish between the first two possibilities. The third one can be separated only by careful examination of serial sections.

Any sharp longitudinal localization of the label will cause a derivation from a Poisson. Such localizations might be: (a) label confined exclusively to the nuclear region; (b) label distributed in the entire cell but with a large concentration in the nuclear region; (c) label restricted to the 600 A granules, i.e., at the poles of the cell; (d) label localized in a small region not related to any recognizable morphological feature. Cases a, c, and d should be easily recognized. Case b presents a more difficult problem, depending on the concentration, and will be examined in the following paper (6).

Radioautography of Cells Labeled with Leucine-H³:

Cross-sections of E. coli 15 T⁻U⁻ fully labeled with leucine-H³ were examined as described above. The grain count distribution is shown in Fig. 9 where it is compared to a Poisson distribution. The agreement between the two is good. Applying the χ^2 test we obtain a value χ^2 = 4.46. For 5 degrees of freedom the probability P that χ^2 have a higher value is close to 0.50, a good fit. The Poisson model is, therefore, acceptable.

For the purpose of comparison with the DNA results we examined the case of cells fully labeled with leucine-H³ and whose morphology had been subsequently modified by growth in chloramphenicol for 60 minutes. The results are shown in Fig. 10. Again, the agreement with a Poisson model is excellent: χ^2 = 6.5, P = 0.30 for 5 degrees of freedom, a good fit.

To test a distribution against a model we must choose between two hypotheses: the null hypothesis
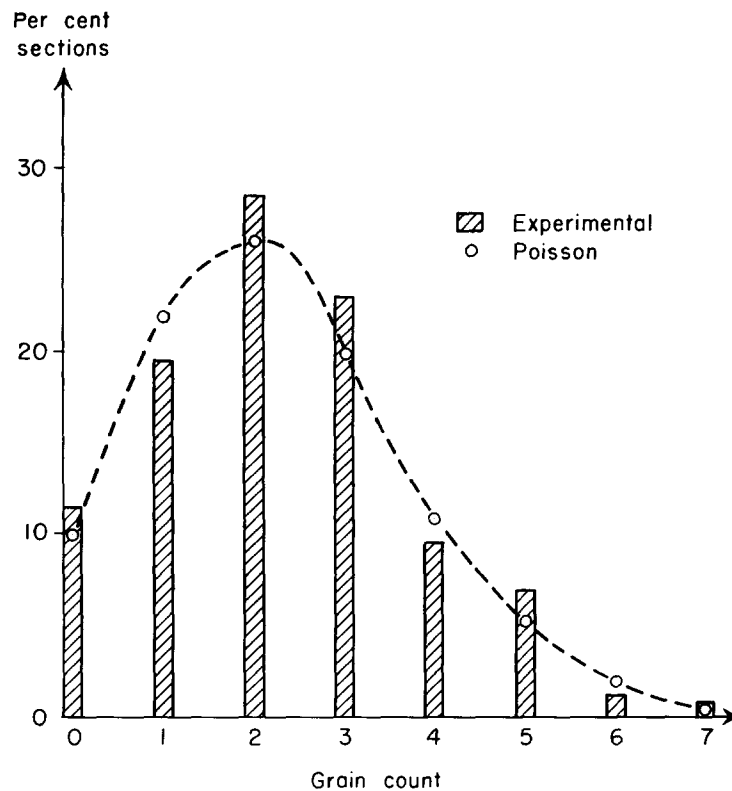

FIGURE 9

Distribution of grain counts over random cross-sections of cells labeled with leucine-H³ (149 sections). A Poisson distribution having same average is also plotted. The fit between the two distributions is good.

according to which the distribution arose by normal variations, due to small sampling, from the chosen model, and the alternative hypothesis, according to which it arose from some other, undefined model. In practice we select a critical set of samples and reject the null hypothesis whenever a distribution falls in this critical set. In our work the chosen critical set was all these distributions for which the probability P that χ^2 be higher than the obtained value was less than 0.05 or more than 0.95. If P, on the other hand, fell between these two values the model was accepted.

For a given value of χ^2 the value of P will depend on the number of constraints placed upon the distribution of sections among the various classes of grain counts. In all our experiments this number is two: one is that the total number of sections is equal to the sum of the sections placed in the various classes, and the other is that the theoretical distribution (Poisson or other) is always calculated from the average grain count obtained from the data. If we have distributed our sections between i classes, this means that the number of degrees of freedom will be $f = i - 2$. Classes with less than 5 sections were grouped together in order to give one class with more than 5. (See reference 31 for more details on χ^2 test.)

It might be of some interest to point out that in the conditions of the experiment the L-leucine-H³ was exhausted in less than 10 minutes. After seven binary divisions and sectioning of the cells each section represents approximately $\frac{1}{1000}$ of the original labeled structure. Yet the distribution of grain counts indicates still a fairly uniform amount of label in each section. This result confirms those of van Tubergen (28) in placing a limit on the largest possible indivisible unit of protein. Identical results were obtained for cells continuously labeled.

Some information regarding the possible sites of protein synthesis was sought by considering the distribution of label after very short exposures to leucine-H³. Fig. 11 shows the grain count distribution obtained with a 1-minute pulse ($\frac{1}{140}$ of the division time). The value of χ^2 found when testing the data against a Poisson distribution was 6.13. For 5 degrees of freedom $P \sim 0.30$, a good fit. Similar Poisson distributions were obtained for the shortest pulse used (42 seconds or $\frac{1}{200}$ division time). Data from serial sections of cells labeled for 1 minute are shown in Fig. 12 (B).
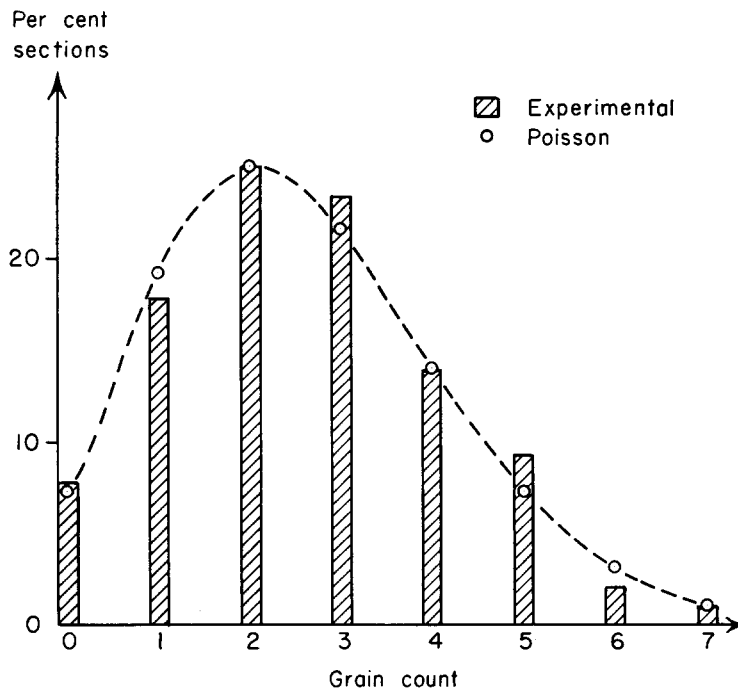

FIGURE 10

Distribution of grain counts over random cross-sections of cells labeled with leucine-H³ and treated subsequently with chloramphenicol (274 sections). The data fit a Poisson distribution.

In general we find thus that cross-sections of cells labeled with leucine, either for a long time or for very short pulses, give a Poisson distribution of grain counts. The most probable interpretation of this is that proteins are evenly distributed within the cytoplasm of the cells (at least when considered with the still fairly coarse resolving power of this method). The data from serial sections in the 1-minute pulse are consistent with this interpretation. They do not indicate an exclusive location of the newly synthesized protein along the cell membrane, for, in this case, the first section through the tip of the cell would have almost the same average grain count as the following ones. This last point is illustrated by Fig. 12 (C) which shows the distribution of grain counts in serial sections of cells labeled with diamino-pimelic acid-H^3 in presence of a high concentration of lysine. In such cells most of the label appears associated with cell wall material (R. P. van Tubergen, personal communication). Only three

sections are shown in this case, as not enough examples of fourth sections were obtained to give a reliable average grain count. Examination of grazing longitudinal sections showed also a higher relative concentration of label in the case of diaminopimelic acid than in that of a leucine pulse.

Radioautography of Cells Labeled with Thymidine-H^3 and Treated with Chloramphenicol: In order to identify more closely the localization of DNA with that of the central, generally lucid region seen in the electron microscope, it was interesting to study the effect upon the localization of DNA of the chloramphenicol-induced shortening of the nuclear region. Fig. 13 shows the distribution of grain counts for 307 sections of cells labeled with thymidine-H^3 and treated with chloramphenicol for 90 minutes. As in normal cells the distribution is obviously not a Poisson, and the difference is here even more pronounced. The value of χ^2, for testing against the Poisson model, is 131.6. For 4
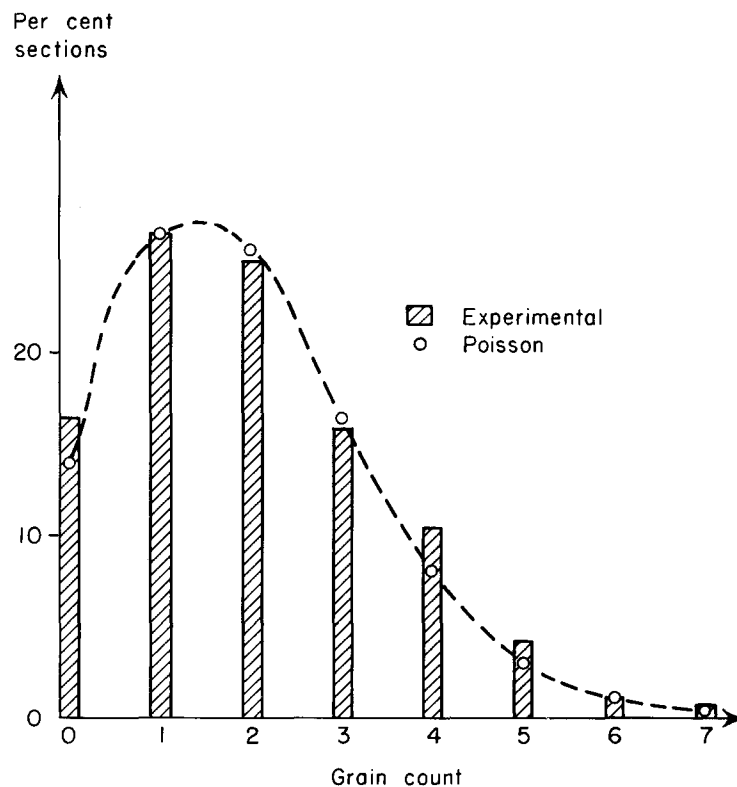

FIGURE 11

Distribution of grain counts over random cross-sections of cells labeled with a 1-minute pulse of leucine-H^3 (340 sections). The data fit a Poisson distribution.

degrees of freedom the probability that χ^2 be higher is $P \ll 0.0001$, an obviously bad fit.

We might make the following assumptions: the label is localized in a region smaller than the whole cell, it is distributed uniformly in that region and, therefore, those sections cutting through that region should show a Poisson distribution of grain counts (an assumption supported by the leucine data). We can then try to determine the size of this region by ignoring the sections having zero grains, fitting a Poisson distribution to the rest of the data and counting the number of sections in the zero class which lie outside that distribution (Fig. 14). We thus obtain the result that 43 per cent of the sections are completely unlabeled.

We then turn to the morphological data obtained by electron microscopy of sections of the same specimen. Examination of a large number of cross-sections revealed that 44.5 per cent of these did not show any nuclear material. Since the sections seen in the electron microscope are thinner than those used for radioautography a small correction must be introduced. When this is done the

number of sections deprived of nuclear material should be 41 per cent. This compares quite well with the figure of 43 per cent sections excluding DNA.

We can also use the electron microscopic data to plot a model distribution on the assumption that the label is contained in the nuclear region (5). Fig. 13 shows that such a distribution fits the radioautography data closely. The χ^2 value is 8.1. For 5 degrees of freedom $P > 0.1$, a satisfactory fit. There is, therefore, good correlation between the distribution of incorporated thymidine-H^3 among sections and the size of the nuclear region modified by growth in chloramphenicol.

Control of Cell Labeling: The last point that should be made is that the variations in the grain count distribution reflect truly the localization of label within the cells and not a non-homogeneous labeling of the cells themselves. Since the organism used is a thymidine-requiring mutant and since the cultures were tested continuously for the absence of back mutations we do not expect the DNA molecules to show uneven labeling. It re

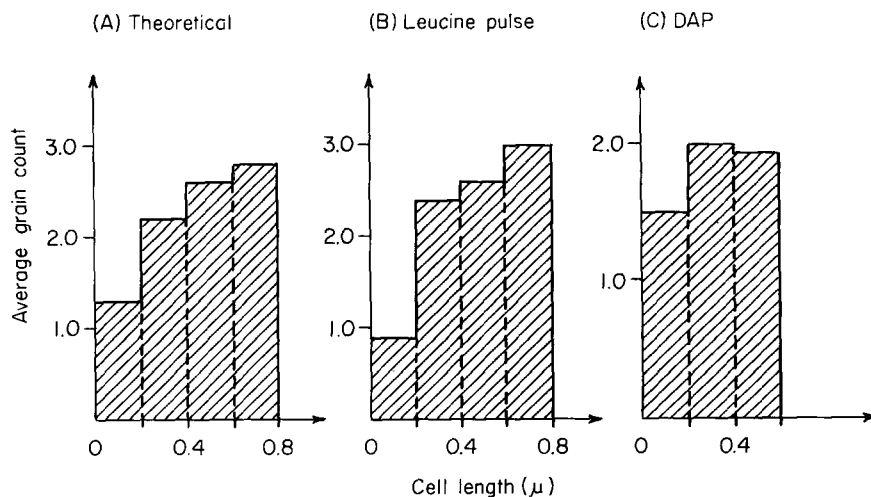(A) Theoretical   (B) Leucine pulse   (C) DAP

Cell length (μ)

FIGURE 12

(A) Expected average grain count distribution for serial sections based on the hypothesis that the label is uniformly distributed throughout the cell. Section volumes have been calculated from electron microscope data.

(B) Average grain count distribution for serial sections of 37 cells labeled with a 1-minute pulse of leucine-H^3. The expected standard deviations are: 0.16 for the first section, 0.25 for the second, 0.26 for the third, 0.39 for the fourth (this last class included only 20 sections).

(C) Average grain count distribution for serial sections of 30 cells fully labeled with diaminopimelic acid-H^3 (DAP) in presence of a high concentration of lysine. The expected standard deviations are: 0.22 for the first section, 0.26 for the second, 0.26 for the third. In this case, a large fraction of the label is associated with the cell wall.

mains to show that cells of equal sizes contain equal amounts of DNA.

It has been shown by van Tubergen (29), using radioautography of whole cells, that *E. coli* 15 T⁻ grown in conditions exactly similar to those used here had incorporated an amount of thymidine-H³ directly proportional to the length of the cell. For each length class the distribution of grain counts was close to a Poisson, showing uniformity of label in cells of similar lengths. Similarly, radioautography on whole cells from aliquots taken before embedding from the cultures used in the above

experiments showed no gross inhomogeneity in labeling. This applied to all the leucine-H³ experiments as well as to the thymidine-H³ experiments.

Another method of checking the homogeneity of labeling involves the use of serial sections. Thus in the case of normal cells grown in thymidine-H³, examined in a previous paper (5), we can select, from the data obtained for serial sections, only those sections which are known from their position in the sequence to cut through the nuclear region. Fig. 15 demonstrates that for these sections the grain counts approximate a Poisson distribution,
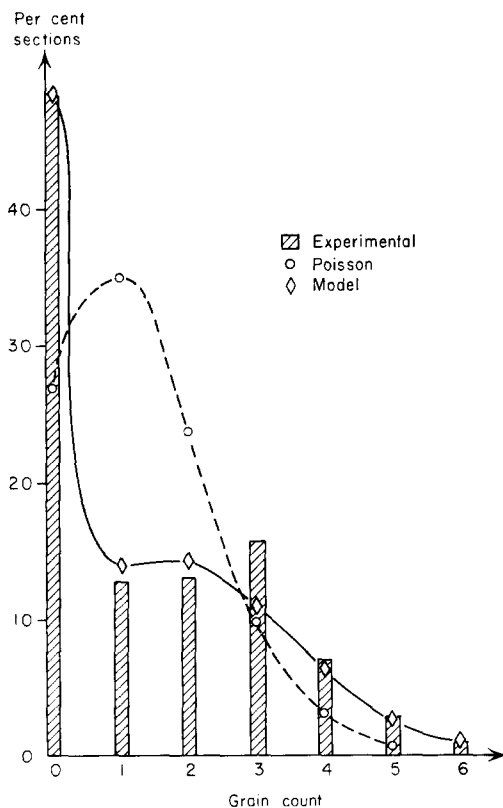

FIGURE 13

Distribution of grain counts over random cross-sections of cells fully labeled with thymidine-H³ and treated with chloramphenicol to modify the nuclear morphology (307 sections). The deviation from the Poisson model is obvious. The model distribution was calculated on the assumption that the label was confined to the nuclear region. It fits the data well.
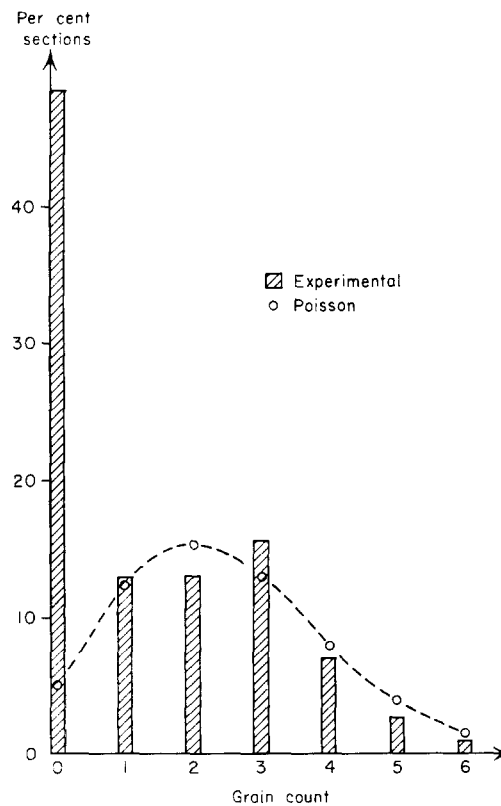
FIGURE 14

Same data as in Fig. 5. A Poisson distribution was fitted to the data, ignoring those sections which had no grain count. It is thought that the number of zero grain sections lying outside this Poisson distribution gives an approximation to the number of unlabeled (purely cytoplasmic) sections.

although the general distribution of grain counts had been markedly different. The χ^2 value is 3.82. For 4 degrees of freedom $P \sim 0.45$, a good fit.

A further, more qualitative check can be made by increasing the exposure time of the radio-autograph to the point in which the average grain count becomes very high and the chance for a labeled section to have zero grain becomes very small. Examination of serial sections of cells labeled with thymidine-H^3, as shown in Figs. 3 and 4, reveals that successive cross-sections through the same cell can show a wide variation in the amount of label contained. The same figures give an example of a longitudinal section showing similar behavior. Although such a technique does not lend itself to quantitative work, since it becomes impossible to count the number of grains associated with the labeled sections, it is illustrative of the fact that the label is localized and is especially useful in clearly demonstrating the site of localization. Furthermore, it is now possible to make a statement as to the possible amount of DNA that might be present in the cytoplasm, if

any. The average grain count above sections cutting through the nucleus, estimated from the length of the exposure, is about 20. That above sections through cytoplasm only is less than 1. In a nuclear section the nucleus represents only approximately $\frac{4}{9}$ths of the total volume. We can thus state that the concentration of DNA in the nuclear region is at least 45 times higher than that in the cytoplasm.

DISCUSSION

The morphological effects of chloramphenicol have been used here as a tool for an experimental modification of the nuclear region and have already been discussed.

The results of radioautographic studies on sections of cells labeled with leucine-H^3 establish the not too surprising fact that within the resolution of the method, proteins are randomly distributed throughout the cell. The situation remains unchanged for cells placed in the presence of chloramphenicol. We have, so far, no way of
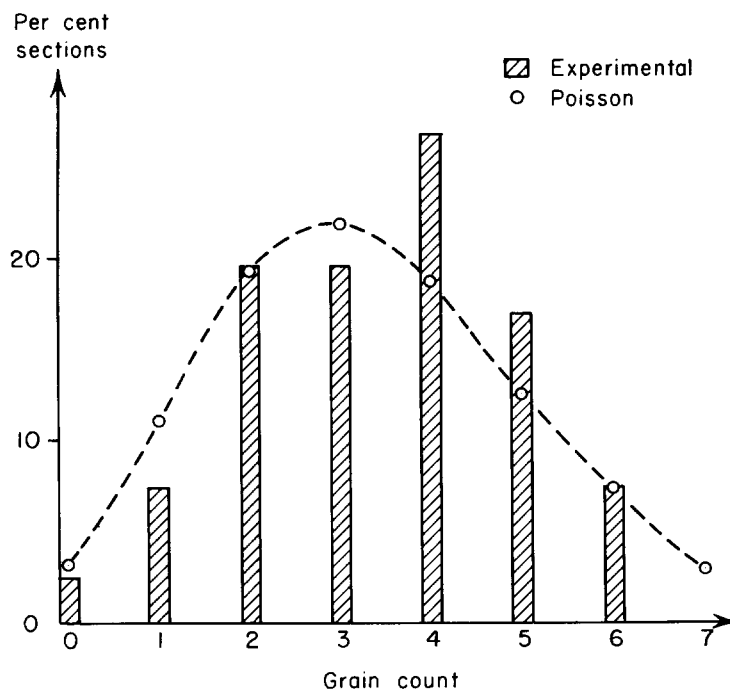

FIGURE 15

Distribution of grain counts over sections cutting through the nuclear region of cells labeled with thymidine-H^3 (41 sections). The slightly higher than usual deviations are probably due to the small sample used. A good fit is indicated by the χ^2 test.

determining whether the nuclear region contains protein or not. This is due to the fact that sections through the nuclear region contain also a sizable amount of cytoplasmic material.

The demonstration that a Poisson distribution of grain counts can be obtained, given reasonably uniform specimens, is not trivial since cases when such a distribution could not be obtained have been reported (17, 12). We were able to obtain Poisson distributions in all the cases of cells labeled with leucine-H^3, diaminopimelic acid (7), and of cells fully labeled with uridine-H^3 or cytidine-H^3 (6) (a total of some 15 separate experiments). This should probably be attributed to the very uniform geometry of the specimens examined and to their thinness, which eliminates almost entirely artifacts due to self-absorption of the beta particles.

In the case of short pulses of leucine (1 minute) we reached the conclusion that the label was randomly distributed and that there was no strong evidence for a concentration of label on the cell membrane. Several hypotheses have been made regarding the sites of protein synthesis in bacteria. On the basis of P^{32} suicide kinetics in recombination of experiments, Pardee (24) has postulated that proteins (specific enzymes) were specified directly by DNA. Our results do not present any evidence in favor of a physical contact between DNA and newly synthesized proteins. The fractionation studies of Butler, Crathorn, and Hunter (4) on *B. megaterium* and of Connell, Lengyel, and Warner (9) on *Azobacter vinelandii* have led them to the hypothesis of an involvement of the cell membrane in protein synthesis. We find no evidence for this, but it must be remembered that the analysis of data from serial sections presents considerable difficulties. Furthermore, McQuillen, Roberts, and Britten (20) have reached the conclusion that a protein unit was made in approximately 5 seconds, and released from its site of synthesis witnin 5 to 15 seconds. The time delays used in the experiments described here (1 minute and 42 seconds) are, therefore, too long to make the results very significant and no final conclusion can be reached from them.

The existence of chromatin bodies in bacteria has been clearly demonstrated—*e.g.*, Robinow (26). Mason and Powelson have shown that similar bodies are visible in living cells (19). Several authors—*e.g.*, Chapman and Hillier (8), Maaløe

and Birch-Andersen (18), Ryter and Kellenberger (27), Murray (21)—have sought to identify the central lucid region seen in electron micrographs with the chromatin bodies. Such an identification was well grounded in circumstantial evidence. Yet it seemed worthwhile to obtain a more direct correlation between the position of the nuclear region and that of DNA.

In an earlier paper (5) we found an association between incorporated thymidine-H^3 and the nuclear region seen in electron micrographs. This has been confirmed here by showing that a morphological modification of the nuclear region was correlated with a change in the localization of the label. The method of analysis was put on a sounder experimental basis by showing that Poisson distributions of grain counts were possible and that derivations from such a distribution were truly due to non-homogeneity of the label within the cell.

Since it is the only morphological feature seen in the nuclear region, it seems probable that the finely stranded material described by several authors (15, 22, 5) represents a DNA complex. The works of Forro and Wertheimer (13), Painter, Forro, and Hughes (23), and van Tubergen (28) show that the division of DNA among the progeny of labeled *E. coli* is not a random process. The apparent lack of organization of the nuclear material at the electron microscopic level is not, therefore, reflected in its behavior during division. It is possible that the specimen preparation procedures have disrupted a morphological organization that was present in the living cell or that some organization is still present but is not seen because of the thinness of the sections. The situation is not very different from that of chromosomes in higher organisms in which the high degree of organization seen in the light microscope is only rarely perceptible at the electron microscopic level.

We hope that we have demonstrated here that radioautographic studies of thin sections of microorganisms can be used to identify sites of label localization. The analysis of grain counts over random cross-sections has revealed, with a fair degree of accuracy, the size of a structure too small to be resolved by radioautography of the whole cell. We should note that such an analysis is especially useful in the cases in which the label is completely restricted to the structure considered. As we shall see in the next paper, cases

in which only part of the label is localized within a structure can be more ambiguous.

Once the size of the label-containing structure has been determined its position in the cell can be found by serial sectioning. It might be thought that serial sections would suffice to give all the information needed. In the case of organisms as small as *E. coli* this is not so because of several factors. It is a much longer, more painstaking, and difficult method of obtaining quantitative information than the use of random sections. Furthermore, the necessity of averaging the data obtained from a number of cells in order to obtain statistically significant grain counts introduced two main sources of error: (*a*) the first section, at the tip of the cell, varies in thickness, and (*b*) the position of the nuclear region with respect to the tip of the cell varies, especially in chloramphenicol-treated cells. These factors are easily taken

into account in the analysis of random cross-sections but not in the case of serial sections. The two methods are therefore complementary.

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