NUCLEAR DIVISION AS OBSERVED IN LIVE BACTERIA BY A NEW TECHNIQUE

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Received for publication August 19, 1955

Ability to demonstrate clearly the sequence of events during division by observing live cells might hasten the settlement of the controversy over the mechanism of bacterial nuclear division. Phase contrast microscopy of living bacteria (Stempen, 1950; Clifton and Ehrhard, 1952) probably provides the best demonstration of the bacterial nucleus in living cells, but these and other studies show the nuclei to have considerably less detail than they have in stained preparations (DeLamater, 1953; Fitz-James, 1954).

A new method for examining dividing bacteria by phase contrast microscopy is described; it demonstrates the structure of bacterial nuclei more clearly than have previous methods and may prove helpful in studying the effects of various agents on nuclear and cellular division. The technique utilizes a principle described by Barer, Ross and Tkaczyk (1953), who observed that phase contrast within a living cell could be altered by changing the refractive index of the medium surrounding the cell. When cells are mounted in a medium with a refractive index near that of the cells, the advantages of phase contrast microscopy are most fully realized. Barer has used concentrated solutions of albumin as a mounting fluid for bacteria. We found gelatin to be more suitable for growing bacteria.

METHODS

Growth of cells. *Bacillus cereus*, strain RS, and *Escherichia coli*, strain 61, were used in the experiments. The cells were grown on brain-heart infusion (Difco) agar slants at 37 C and were transferred every 24 hr for at least 3 days prior to use. A 24-hr culture was inoculated into 15 ml of brain-heart infusion broth in a culture tube and incubated at 37 C for 6 hr. About 0.2 ml of this culture was spread uniformly over the surface of a plate of brain-heart infusion agar and incubated at 37 C for 1½ to 2½ hr.

Examination of living cells in gelatin. Coverslip impressions from cultures on brain-heart infusion agar were mounted in concentrations of gelatin from 20 per cent to 40 per cent by weight in brain-heart infusion broth. A more satisfactory method utilized cells that were grown at 37 C for 2 to 4 hr in small serology tubes containing 5 to 8 ml of the gelatin brain-heart infusion broth. A drop or two of the actively growing culture was placed on a slide, covered with a sterile coverslip, and sealed with melted parawax.

Concentrations of gelatin used were: from 20 to 27 per cent with *E. coli* and from 28 to 35 per cent with *B. cereus*. The concentration for the best results could only be determined experimentally. The gelatin was prepared by adding the dry gelatin (Difco) to the proper amount of hot (90 to 100 C) brain-heart infusion broth. The mixtures had to be steamed for periods up to an hour to get the higher concentrations of gelatin into solution. The solutions were steam-sterilized for 15 min at 121 C. The gelatin solutions were centrifuged at 37 C for 1½ hr at about 3000 × G in order to clarify them for microscopy. Cell division was followed by phase contrast microscopy. Because the cells grew more rapidly and because the gelatin solutions were more manageable at 37 C than at lower temperatures, all manipulations, observations and photography were carried out at 37 C.

Fixation. When cells were fixed, agar blocks from the agar plate cultures were placed in the vapors of 2 per cent osmium tetraoxide for 1 to 5 min. Cells were also fixed by placing the agar blocks in the vapors of formalin (40 per cent formaldehyde) for 10 to 30 min and then treating the fixed cells with 1 per cent acetic acid.

Coverslip impressions of the fixed cells from the surface of the agar blocks were mounted in distilled water on a slide. The cells could be washed, hydrolyzed and stained by flooding the proper reagent under the coverslip.

[1] National Science Foundation Predoctoral Fellow. Submitted by senior author in partial fulfillment of requirements for the degree of Master of Science.

Fixed cells were examined by phase contrast microscopy, and the same cells could be observed during and after hydrolysis. After hydrolysis with 1 N HCl for 1 to 1½ hr at room temperature (23 to 28 C) and washing with distilled water, the cells were usually stained with 0.1 per cent basic fuchsin for 1 to 5 min. The stained cells were examined by ordinary light microscopy.

Microscopy. All observations were performed using a Spencer phase contrast microscope with a turret condenser. The 97 × bright high contrast oil immersion (1.25 NA) was the most satisfactory objective. This objective has not been used extensively heretofore, according to the literature. It provided better contrast of small details than did the B minus or bright medium contrast objectives. Photographs were taken using a 35-mm Praktiflex camera with either Adox KB 14 or Kodak Microfile film. Either 12.5 × or 15 × oculars were used. The magnification on the film was maintained at 1500 ×. A Wratten filter #58 was used to improve contrast.

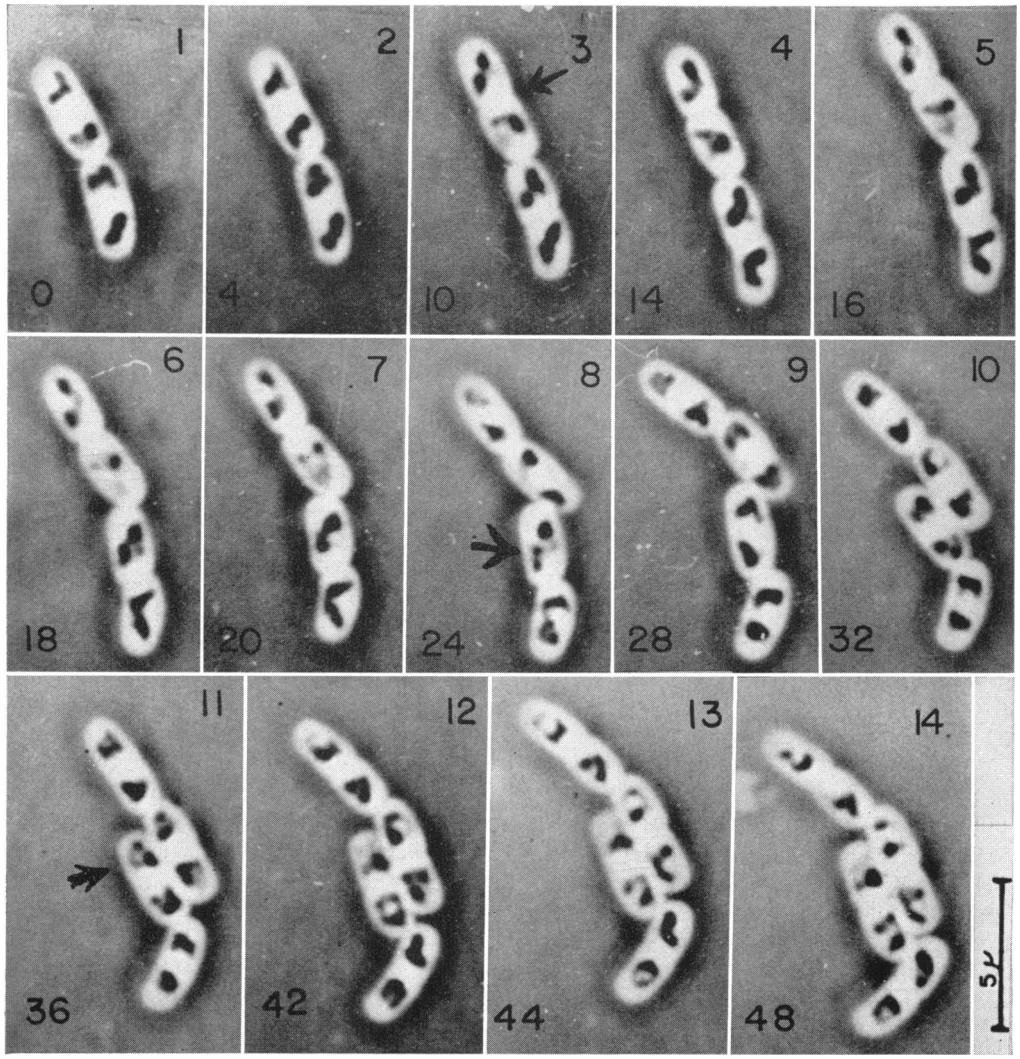
RESULTS

Mounting young cells in gelatin solutions rather than in broth reveals more of the internal structure. The areas of low refractive index, which cytologists have identified as the nuclei in young, growing bacteria, are sharply in contrast by this technique. With the bright high contrast objective these areas are darker than the rest of the cell and thus in photographs appear similar to nuclei of stained preparations. Under conditions used in these studies concentrations of gelatin from 20 to 27 per cent with *E. coli* and 28 to 35 per cent with *B. cereus* provided good contrast. The lower concentrations usually revealed the internal structure more clearly. Both of these species grew well in the gelatin brain-heart infusion media. The structures that were observed in the cells did not arise from an effect of the gelatin on the cell. These structures were observed as soon as the cells were mounted in the gelatin medium and also in mineral oil. They were seen in cells growing in brain-heart infusion broth but could not be revealed in photographs as clearly as in cells mounted in the gelatin medium.

By this method it has been possible to photograph more of the detail in the dividing nucleus than has previously been shown in live bacteria. Several photographic records have been made to follow the sequence of the morphological changes in dividing bacteria. *E. coli* was the subject in most of these. The nuclear configurations that appeared during the divisional sequence were similar in every cell that was studied. Also, a similar pattern of cell division was observed in these cells. Some photographs from a sequence are shown in figures 1 to 14.

The sequence of events during division of *E. coli* appears to be as follows. After the separation of sister nuclei (figure 1 or 10), a septum is formed and for the first 6 or 8 min of this period the nuclei seem to remain quiescent, appearing as compact, 3-lobed structures. As the sister cells begin to separate (evidenced by indentations, figure 3), the nuclear figures change, appearing more distinctly lobed or elongated, depending upon their 3-dimensional positions in the cell. The 3 lobes seem to move apart somewhat but are connected by fine strands of a material having a similar optical density (figures 4 and 11). Then, the individual lobes of the nuclear element divide but remain connected (upper pair, figures 5 to 7; lower pair, figure 8). At this stage 4 or 5 lobes have been observed, very definitely in some sequences, but 6 have never been made out distinctly. The nuclear multi-lobed structure pulls apart and the 2 parts may appear as single large masses (lobed elements in side view?) or as 2- or 3-lobed structures which remain connected for a time by what appear to be fine strands (figure 8). The sister cells usually become clearly separated now. Within a few minutes two separate, 3-lobed elements occupy opposite poles of each enlarged cell of the pair, and then indentations suggest septum formation and the beginning of the separation of the new sister cells (figure 11).

Under the growth conditions used in this study the generation time of *E. coli* is about 30 min. Except for perhaps 6 min of this period the nuclei are undergoing the movements associated with their division. The pulling apart of the sister nuclei takes place in a relatively short time—6 to 8 min. Since the cell wall of *E. coli* could not be seen, it has not been possible to establish when the septum completely separates the sister cells, but indentations suggesting the separation of the cross walls occur within 6 to 8 min after the nuclei have divided. The sister cells separate as individuals about 12 min later. At this time each cell contains a large nuclear element that proceeds to divide within the next 12 min. Thus, if doubling of the viable cell count were used as an index of

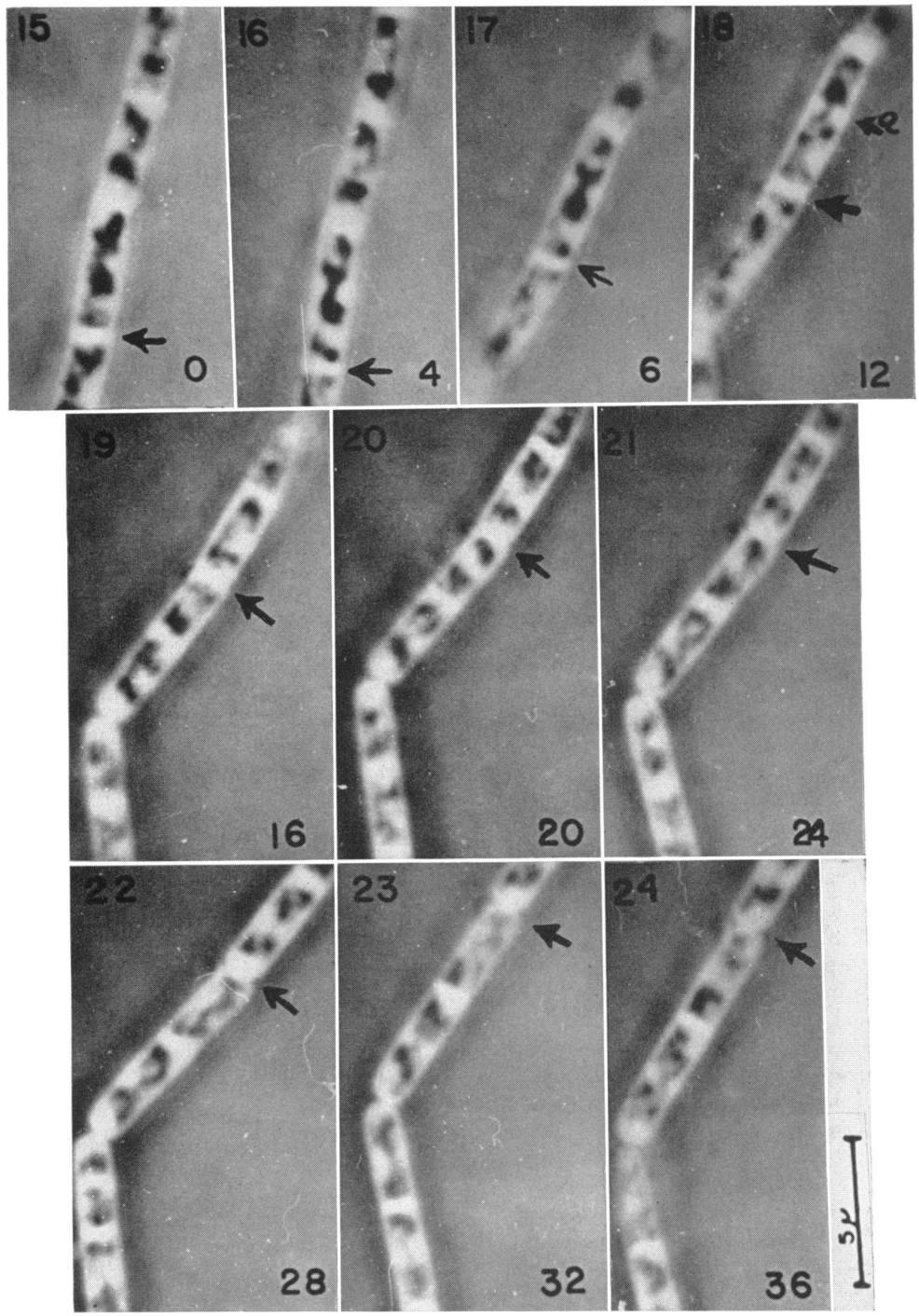


Figures 1 to *14. Escherichia coli* 61 growing in 20 per cent gelatin brain-heart infusion medium. Age of cells in minutes in lower left corner of each photograph. Bright high phase contrast microscopy.

the completion of cell division, the separation of the nuclear elements would appear more closely to follow than to precede this event. Lark and Maaløe (1954) using viable counts concluded that in *Salmonella typhimurium* nuclear division followed cell division.

Cells of *B. cereus* mounted in the gelatin medium showed a similar increase in contrast and detail. However, the nuclear structures in this organism appeared more complex than those in *E. coli*. It is more difficult to interpret the sequence of events during division in this species (figures 15 to 24). In many instances the edges of the forming septum interfered with the picture by overlying the nuclei (*a* in figure 18, for example). Often the septum appeared to be forming across the area where the nuclei were still in the process of separation. The cell wall and septa could be clearly demonstrated in *B. cereus* by using gelatin concentrations 1 to 3 per cent higher than those used to show the nuclei (figure 25). This technique could not be modified in any way to show the cell walls of *E. coli*.

Photographs of dividing cells were taken usually at 2-min intervals. A few series were made at minute and half-minute intervals. These revealed



Figures 15 to *24. Bacillus cereus* RS growing in 32 per cent gelatin brain-heart infusion medium. Age of cells in minutes in lower right corner of each photograph. Bright high phase contrast microscopy. Arrows follow one cell septum.

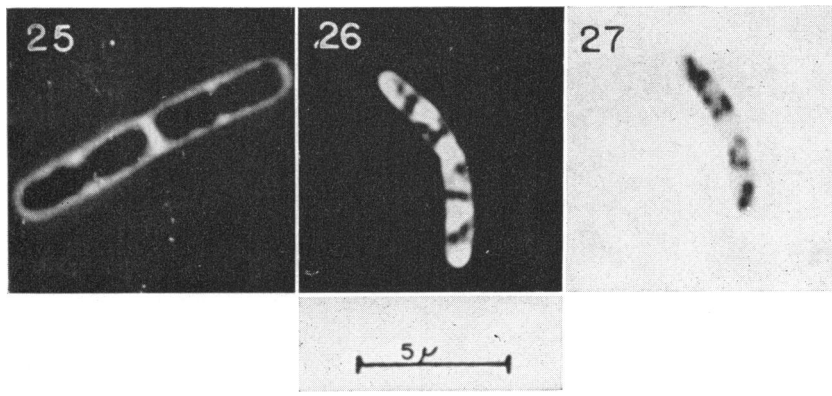


Figure 25. Live *Bacillus cereus* RS mounted in 33 per cent gelatin to demonstrate cell wall and cell septa. Bright high phase contrast microscopy.

Figure 26. Escherichia coli 61 fixed 4 min in the vapors of 2 per cent osmium tetraoxide then mounted in distilled water. Bright high phase contrast microscopy.

Figure 27. Same cell as in figure 26 after hydrolyzing in 1 N HCl at R.T. for 60 min and then staining 1 min with 0.1 per cent basic fuchsin. Ordinary light field microscopy.

no more of the pattern of nuclear division than the photographs that have been used here to show typical changes during a division in *E. Coli* and in *B. cereus*. Occasionally the division of the lobes of the nuclear element could be observed in greater detail but just how these elements finally separate could not be demonstrated.

Though it seems unlikely that areas consistently showing certain configurations associated with division are not nuclei but inclusions of some sort, supportive evidence of their nuclear nature was sought from data provided by studies on fixed cells. In a preliminary survey a single living cell in broth or water was photographed and then fixed and photographed again. The nuclear area revealed by phase microscopy rather indefinitely in the living cell remained in the same location and with the same configuration as a clearly defined area in the fixed cell. Thus, fixation did not create an artifact but did improve the contrast of these areas in cells suspended in water or broth (Tulasne, 1950; Mason and Powelson, 1955). In the fixed cells the configurations of the areas of low refractive index were strikingly similar to those in live bacteria mounted in gelatin and those of stained preparations reported by DeLamater (1953), Fitz-James (1954) and others. When a fixed cell (figure 26) was hydrolyzed with 1 N HCl for 1 to 1½ hr at room temperature and stained with basic fuchsin (figure 27) the stain always localized in the areas that were of low refractive index in the fixed cells.

DISCUSSION

The use of high concentrations of gelatin to bring the refractive index of the background nearer that of the cytoplasm of growing bacteria has revealed a well-defined nuclear figure in untreated living cells that is similar in structure to that of the fixed or stained cell. Since the image formation in this technique is mainly due to local differences in refractive index in the cell and has no cytochemical specificity, it is important to know whether the areas of low refractive index represent only nuclear material and whether the areas of higher refractive index are all extranuclear material. It was not possible to photograph the same cell mounted in the gelatin medium before and after fixation, and we are unable to assert positively that the configuration of the area of low refractive index in this cell would exactly coincide with the same area in the fixed cell. However, in the living cell mounted in water or broth the location and configuration of this area do not change when the cell is fixed, and the area of low refractive index in the fixed cell is what stains after hydrolysis. (The lack of contrast in the photograph of the living cell in broth does not permit publication from a print.) The Feulgen reaction in these areas is too faint to give conclusive data. The areas of low refractive index possibly constitute more than the location of desoxyribonucleic acid; they may be composed of protein serving as a matrix or support for nucleic acids and other materials.

The configuration of these areas was shown, in

the case of *E. coli*, to change in an orderly and reproducible fashion during division. The interpretation of these figures in terms of a basic pattern of division is restricted by several factors. For one thing only a small area of the cell is in focus at one time. All pictures of a sequence should be taken at the same level of focus. However, because a refocusing on the cell must be done every few seconds, one cannot be certain that the adjusted focus is always at the same level in the cell. We observed that the nuclear configuration could change depending upon the level of focus. For that reason, some of the apparent changes of the nuclear structures between intervals in a sequence may be due only to slight differences in focus. Similarly, the differences in their 3-dimensional position in the cell affect the appearance of the nuclear areas. In the case of *B. cereus* perhaps certain inclusions as well as the septa complicate the picture. After consideration of the configurations in sequences that have been obtained we have concluded that it is not possible to discern from these data the pattern of nuclear division. Some indications for a pattern have been described in a discussion of figures 1 to 14 in the section on results. Among all of the records no evidence could be found for the occurrence of mitotic figures *in a sequence typical of nuclear division* in various plant and animal cells.

Despite its limitations there are several advantages that this technique has over other methods for demonstrating bacterial nuclei. It is time-saving and permits a view of a natural sequence rather than an artificial one. We believe that variations of this procedure may provide the means to accumulate further data on the cytology of phage infection, sporulation and germination, and the effects of toxic materials on nuclear and cellular division.

SUMMARY

A method is described that uses high concentrations of gelatin to increase the phase contrast within living bacteria. The sequence of division of cells of *Escherichia coli* and *Bacillus cereus* has been photographed. The nuclear areas in these dividing cells appeared to be as clearly defined as the areas in fixed and hydrolyzed and stained cells. However, a pattern of nuclear division could not be formulated from the sequences studied.

ADDENDUM

Swellengrebel in 1906 observed similiar 3-lobed and also spiraled configurations of nuclear elements in a large bacillus (probably *Leptotrichia*) in fixed and stained preparations. He also used a special staining procedure that showed the initial stages of septum formation. His drawings are amazingly similiar to various photographs that we have obtained during our study.

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