THE STRUCTURE, DIVISION, AND FUSION OF NUCLEI OBSERVED IN LIVING CELLS OF MYCOBACTERIUM THAMNOPHEOS

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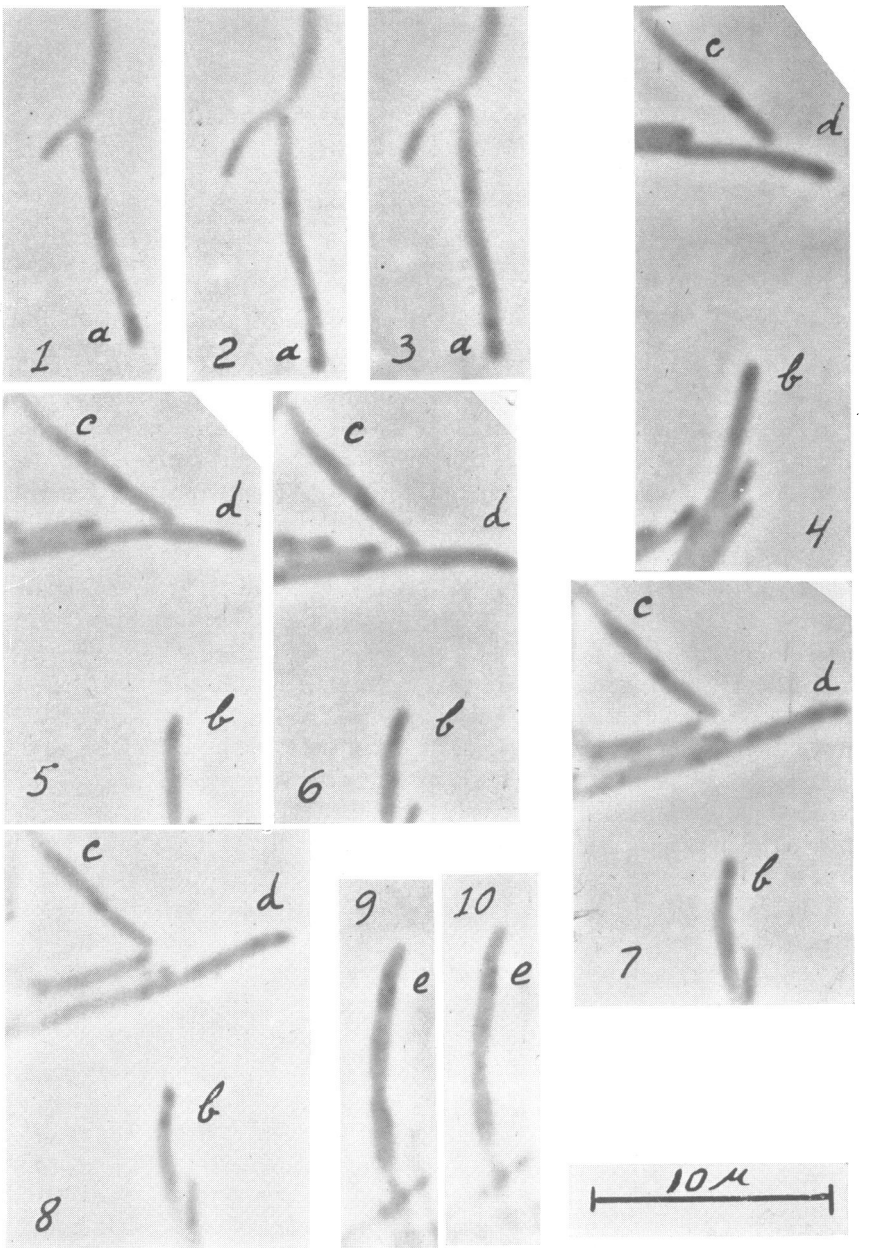
Of all the criteria of the cell nucleus none are more significant than division and fusion when these are associated with definite reproductive processes, or when the dividing or fusing body has the characteristic structural organization of a nucleus. Unfortunately, however, neither division nor fusion is easily recognizable or differentiable from each other in stained preparations (see Knaysi, 1951, pp. 82 to 83, and 108 to 111). Since the development of the phase microscope, nuclei and nuclear division have been observed in living cells of certain bacteria (Tulasne, 1949; Knaysi, 1951, 1952). Published photographic records show that the granules of *Mycobacterium tuberculosis*, which according to Knaysi, Hillier, and Fabricant (1950) are the cell nuclei, appear dark, indicating a greater optical density than the surrounding cytoplasm, but the nuclei of the other species investigated appear as bright bodies in a dark cytoplasm. However, with the exception of the forespore nucleus of *Bacillus cereus*, in which chromosomes, nucleoplasm, and a nuclear membrane have been clearly seen (Knaysi, 1952), no clear-cut organization has been revealed within these nuclei. As far as the author knows, no one has yet reported observing nuclear fusion in living bacteria although several interpreted the giant nuclei one observes in certain stained cells as fusion nuclei (Badian, 1933, 1935; Kleineberger-Nobel, 1945; Bisset, 1948*a*, *b*, 1949; Cassel, 1951).

In the course of a further investigation of the granules of *Mycobacterium*, it was decided to observe, if possible, the behavior of these granules during growth and reproduction. We employed *Mycobacterium thamnopheos*, a culture of which was kindly sent to us by Dr. Stuart Mudd and Mr. L. C. Winterscheid.

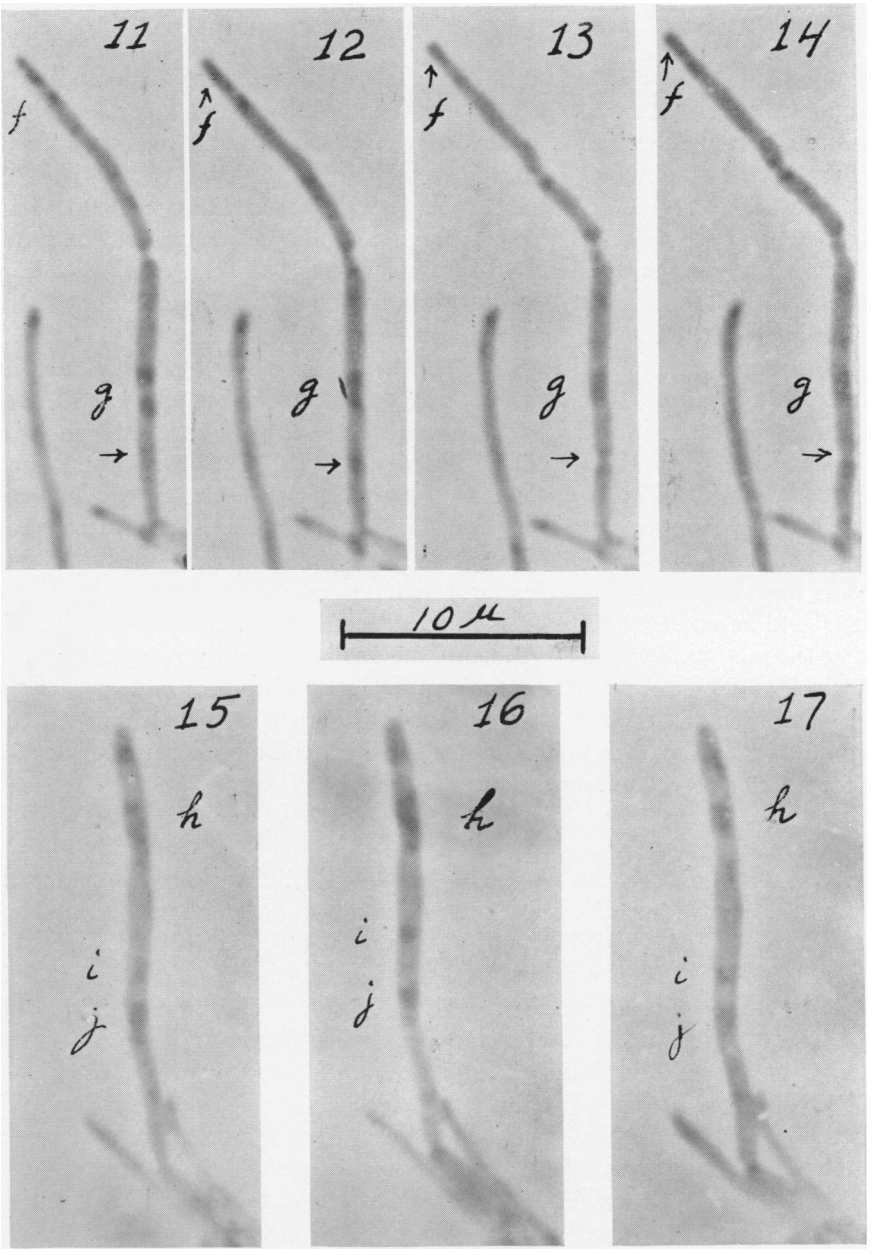
The technique of preparing microcultures where various structures and activities of bacteria may be observed under excellent optical conditions was described by Knaysi (1940). Two modifications are introduced in the present work: (a) A sterile cover glass is placed in position while the loopful of agar is still fluid and left until the agar hardens; this cover glass is slid off then, leaving a disk of agar of the proper thickness. (b) Before the culture is sealed, a capillary glass tube of an external diameter not exceeding the thickness of the chamber is inserted under the final cover glass so that it protrudes about 5 mm outside the boundary of the chamber. When the chamber is sealed, this capillary allows gaseous exchange between the chamber and the environment without endangering the purity of the culture or causing noticeable drying of the chamber within several days. The agar medium used had the following composition: Bacto agar (Difco), 2 g; tryptone, 0.5 g; glucose, 0.5 g; beef infusion diluted with an equal



Figures 1 to *8*. Serial photomicrographs showing the division of a polar granule *a*. Age of microculture: 1, 17 hr + 30 min; 2 and 3, 18 hr + 10 min; 2 focused on granules other than *a*. Temperature 29 to 31 C.

Figures 4 to *8*. Serial photomicrographs showing the division of granules *b*, *d*, and the fusion of two small granules *c*. Age of microculture: 4, 23 hr + 30 min; 5, 24 hr + 20 min; 6, 26 hr + 15 min; 7, 29 hr; 8, 31 hr. Temperature 26 to 28 C.

Figures 9 and *10*. Serial photomicrographs showing the structural organization of granule *e*. Age of microculture: 9, 29 hr; 10, 31 hr. Temperature 26 to 28 C. Note the two pairs of connected bodies (chromosomes). The nuclear membrane, clearly visible in the illustrated material, is not made out readily in the figures.



Figures 11 to *14*. Serial photomicrographs showing fusion of the polar granules *f* and the central granules *g*. Note in the same cell containing *g* that another granule (arrow) has divided and its division was followed by cell division. Note in 11 that this granule contains two parallel dumbbell-like bodies and in 12 that division takes place across the handles of the dumbbells. Age of microcultures: 11, 23 hr + 25 min; 12, 23 hr + 40 min; 13, 24 hr + 10 min; 14, 24 hr + 40 min. Temperature 26 to 29 C.

Figures 15 to *17*. Serial photomicrographs showing the structural organization of granule *h*. Note in 15 and 16 what appear to be three pairs of connected bodies (chromosomes). The nuclear membrane, clearly visible when directly observed, is difficult to make out except in figure 17. Age of microcultures: 15, 16 hr + 30 min; 16, 17 hr + 30 min; 17, 18 hr + 30 min. Temperature 26 to 28 C.

volume of distilled water, 100 ml. The microcultures were incubated at room temperature and observed in dark contrast with a phase microscope manufactured by the American Optical Company. Photographic records were taken with a Contax camera on 35 mm Panatomic-X film.

Observations typical of numerous experiments are recorded in figures 1 to 17. Figures 1 to 3 show the division of a terminal granule *a*. Figures 4 to 8 show the division of terminal granules *b*, *d*, and the fusion of two small granules *c*. Figures 11 to 14 show the fusion of terminal granules *f* and central granules *g*. Figures 9 and 10 and 15 to 17 show the structural organization, respectively, of two large granules *e* and *h*, and some details in the division of a small granule *i*, and in the fusion of granules *j*. Granules *a* to *d*, *f* and *g* had also a similar organization, but we did not succeed in recording the details of that structure photographically. As clearly seen in granules *e* and *h* of figures 9, 10, and 15 to 17, a granule usually consists of an outside membrane enclosing a ground substance in which one finds one, two, or three pairs of minute bodies, each pair having the shape of a dumbbell. In old cultures one occasionally finds a granule in which the minute bodies are single and in active brownian motion; more frequently, single or dumbbell-like bodies are absent and, in optical section, one sees a uniform ring lining the section of the outside membrane as illustrated by Knaysi, Hillier, and Fabricant (1950) for *M. tuberculosis*. This latter structure is characteristic of resting cells, and we often observed the transformation of a granule from one containing dumbbell-like bodies to one in which these bodies are replaced by a uniform line as a cell enters the state of rest.

DISCUSSION

The granules of *Mycobacterium* have been considered variously as spores, lipoid inclusions, volutin inclusions, vacuoles of unknown nature (reviewed by Knaysi, 1929), nuclei (Knaysi, Hillier, and Fabricant, 1950), and mitochondria (Mudd *et al.*, 1951). The present work shows that, regardless of size or location, they have the structure and behavior characteristic of nuclei. We have also ascertained that, regardless of size or location, they give staining reactions given by nuclei, and by the appropriate staining procedure show a structural organization similar to that observed in the living state. In most cells the Feulgen reaction is faintly positive, but here and there one sees granules, often terminal, giving very strong reactions and having organization similar to the one described previously.

The frequency with which fusion of these nuclei takes place is surprisingly high. Indeed, pictures like that of cell *g* of figures 11 to 14 suggest that the division and fusion of nuclei may take place simultaneously in the same cell. While nuclei *g* are fusing, the nucleus indicated by the arrow, which shows two dumbbell-like bodies in figure 11, divides across in figure 12, and this is followed by division of the cell in figures 13 and 14. The genetical implications of this phenomenon should be highly significant.

We have not observed what may be unequivocally interpreted as fusion of cells. The dark, cross line between *i* and *j* in figure 16 seems to have disappeared in figure 17, suggesting fusion between sister cells in the manner described by

Schaudinn (1902). However, we do not consider the evidence sufficiently solid as a basis for conclusions. The cytoplasmic bridge between cells *f* and *g* in figure 12 may well have appeared in stained preparations as a copulation tube, but the sequence of its development in figures 13 and 14 precludes such an interpretation. It is shown that a small, terminal nucleus of cell *g* adjacent to the cytoplasmic bridge divides (figure 11) and one of the sister nuclei enters the bridge (figure 12) and, with the bridge cytoplasm, is separated from cell *g* and the daughter cell of *f* as a small, independent coccus cell about 0.4 μ in diameter (figure 13). The minute cell grows so that in figure 14 it has a diameter of about 0.7 μ. The diameter of cell *f* is about 1 μ.

Several investigators observed in stained preparations of spore-forming bacteria elongated nuclei that they interpreted as "fusion nuclei" and concluded that the endospore is a sexual spore. The author (Knaysi, 1951) has demonstrated that large, elongated nuclei may indicate growth and division, and the present work shows that a fusion nucleus does not have to be very large. It is probable, nevertheless, that nuclear fusion as demonstrated in *M. thamnopheos* also takes place in other bacteria, including spore-forming ones, and that some of the "fusion nuclei" of the literature are genuine. However, since the present investigation shows that nuclear fusion takes place in some nonspore-forming bacteria with remarkable frequency, it does not seem permissible to conclude, without further evidence of significance, that the endospore is a sexual spore. It is possible that the recently observed division of the nucleus within the forespore (Knaysi, 1952) indicates a diploid state of the initiating nucleus, but such a division does not appear to be general since many germinating spores contain only one nucleus each.

SUMMARY

Serial photomicrographs of growing microcultures, taken with the phase microscope in dark contrast, show that the granules of *Mycobacterium thamnopheos* divide and fuse and have the structural organization typical of the bacterial nucleus. They also have the staining properties of nuclei. Their Feulgen reaction is generally faint, but here and there granules giving a strong reaction may be seen. The fusion of nuclei occurs with a surprising frequency and may even be observed in cells where other nuclei are dividing. The formation of a small, terminal coccus cell is described and illustrated.

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