THE MORPHOLOGY OF THE MYCOBACTERIA[1,2]

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The occurrence of branching forms of acid-fast bacteria was first emphasized by the classification of this group of microorganisms by Lehmann and Neumann in 1896 in their family "Hyphomycetes" and genus "Mycobacterium." Such a classification was based on the observation of supposedly filamentous and branched forms by Nocard and Roux (1887), Metschnikoff (1888), Maffucci (1892), Coppen Jones (1895), and Bruns (1895). These morphological studies were all made on organisms stained with carbol fuchsin. A minute accuracy is not possible under these conditions, since apparent branching may be due to a hazy outline of the cell boundaries; to capsular material staining continuously with the cell wall; and to the destruction of stereo-relationships so that it cannot be determined whether a cell lying at right angles to another cell is a branch on that cell or merely lying beside, underneath, or above it.

Miehe (1909) was the first to watch acid-fast bacteria developing in broth microcultures. He concluded that a saprophytic form, the "Harn" bacillus, did not branch, but that the human tubercle bacillus branched by budding, occasionally. In recent years controversy over the mode of reproduction of the human tubercle bacillus has stimulated several bacteriologists to watch acid-fast bacteria growing. Kahn (1929) believes that under given conditions—namely, single cells multiplying in microdroplets of a synthetic medium—the human tubercle bacillus reproduces by

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division into granules. These granules "sprout" into "delicate" rods, which in turn develop into "mature" rods. Gardner (1929) reports that human tubercle bacilli growing on serum agar blocks divide by binary fission only. Oerskov (1932), also using agar blocks and working with *M. phlei* and *M. tuberculosis*, says that the Mycobacteria are actinomycetes because they sometimes form a mycelium which later divides into segments or show an angular type of growth with frequent branching. Jensen (1934) divides the Mycobacteria into two subgenera on the basis of mode of cell division as observed by direct agar-microscopy, the one group, A, identified by the "snapping" and "slipping" growth, the other, B, exhibiting the "snapping" growth only. The "snapping" growth is the same as Oerskov's "angular" growth, and is defined by Jensen as follows: "a cell grows to a certain length, a line of division is formed at the middle, and the daughter cells bend suddenly into an angle, thereby producing V- or L-shaped figures. . . ." The following is his description of "slipping" growth: "after division, the ends of the daughter cells bend, slip past each other, and grow into parallel bundles." True branching occurs sometimes in the early stages in subgenus A; and there is a "transformation from branched rods to cocci" as growth progresses in subgenus B.

Micromotion pictures of the development of several members of the Mycobacteria, including *M. phlei*, tubercle bacilli from cold-blooded animals, and a non-acid-fast strain, have been made by Wyckoff and Smithburn (1933) and Wyckoff (1934a and b). Their findings are that these organisms multiply by binary fission in the active stages of cell division, that cocco-bacilli may be formed in aging microcultures, and that branching rods may be frequently found in cultures of the smooth types and occasionally in rough type growths.

Thus a survey of the literature leaves the reader with no clear-cut conception of a morphology characteristic of the members of the genus Mycobacterium. The factor preventing the organization of such a concept from the work of the various authors, is that the studies have been made using different methods of observation and under conditions for growth differing in respect to the

culture medium, moisture, and atmospheric oxygen. The personal factor of varying interpretations of similar observations, necessarily involved in microscopic work, also leads to confusion.

In isolating single cell strains of acid-fast bacteria by means of microcultures, we became interested in the growth phenomena; and in an attempt to clarify our own morphological concepts, made the following observations.

METHODS

Manner of mounting microcultures. The microcultures were made on thin number 1 coverslips mounted on hollow ground slides. The coverslips were cleaned with acid alcohol, and a thin coating of grease applied by dipping in a solution of vaseline in gasoline. They were then heated in a hot air oven at 160°C. for one hour, again cleaned in acid alcohol, and sterilized by heating in the oven at 160°C. for four hours. After this treatment there remains on the coverglass a very thin but adequate film of grease, the function of which is to prevent the droplets spreading. Droplets of glycerol broth were deposited around the edge of the depression of the slide in sufficient quantity to maintain saturated moisture conditions and in such a manner that there was no interference with the vision of the inoculated droplets. By proper regulation of the amount of grease on the coverslips and of the moisture conditions, the edge of the droplets will remain entire for several weeks. The coverslips were sealed on the slides with vaseline when incubation was carried out at room temperature, and with a half and half mixture of vaseline and paraffin when incubation was at 37°C.

Method of observation. Growth of individual organisms was followed by microscopic observations at such intervals of time as were necessary, depending upon the rate of division of the species under examination. A 1.8 mm. oil immersion objective and a 15× hyperplane ocular gave magnifications of 1425 times.

OBSERVATIONS

Culture. Mycobacterium 5-3A, smooth type. Mycobacterium 5-3A was isolated at the University of Wisconsin from the mesenteric lymph

node of a cow, but is non-pathogenic for rabbits, guinea pigs, and chickens.

Medium. Mannitol synthetic liquid medium (Dorset's formula with 1 per cent mannitol substituted for the glycerol[3]).

Method. By means of micropipettes controlled with a Chambers' micromanipulator, microdroplets of the medium containing one bacterial organism each were laid down in a circle around a loopful of the medium in the center of a coverglass. The bacterial suspension from which the micropipettes were filled was prepared from a forty-eight-hour culture of the bacteria in the mannitol synthetic broth. The slides were incubated at room temperature which varied from 22 to 25°C.

Microscopic examinations were made at intervals of twenty minutes. Since the division time of this organism is about five hours, the periodic examinations permitted following the fate of an organism and each of its daughter cells for approximately twenty-four hours, after which the number of organisms became so large as to cause confusion to the observer.

Data. Plate 1, figures 1 through 5, are representative of the results obtained. These particular drawings were selected from those depicting the development of about 50 single cells, as illustrating the points we wish to emphasize. Drawings did not happen to have been made in this case from the single cell stage up to the nineteenth hour, although observations were taken from the time of planting the single cell through the twenty-fifth hour at fifteen-minute intervals. We have deduced the following principles from these observations:

1. The organisms divide only by binary fission in young cultures.

2. The cells divide into two approximately equal parts. These two cells may, however, grow in length at different rates, but eventually attain about the same length. See cells 1*a* and 1*b* in figures 2, 3, and 4, and cells 4*a* and 4*b* in figures 2, 3, 4, and 5.

3. The rate of division depends upon some factor other than age and size of cell. See cell 7 in figure 2, and cells 7*aa*, 7*ab*, 7*ba*, and 7*bb* in figure 3. As corollaries to this principle, (a) rods of unequal size may divide. See cells 2 and 4 in figure 1, and 2*a*, 2*b*, 4*a*, and 4*b* in figure 2; (b) individual rods vary greatly in length (from 1.5 micra to 4 micra as shown by micrometer measurements).

[3] K_2HPO_4 ..... 1.0 gm. Ferric citrate..... 0.063 gm.
Tri-sodium citrate ..... 0.5 Asparagine..... 5.0
$MgSO_4 \cdot 7H_2O$ ..... 1.0 Glycerol..... 70.0 cc.
Dilute to 1,000 cc. with distilled water.

4. The cells grow in chains, the coherence apparently being due to the presence of capsular material. This capsular material can be demonstrated in macrocultures by Barlow's method of staining gum, as described by Fred and Waksman (1928).

5. The individual cells vary in shape. This seems to be due to two factors, (a) the cells are flexible (the idea of a rigid cell wall is apparently not true for young cells), and (b) as the cells grow in length, crowding occurs in the chain and due to the growth pressure the shape of the organism changes. These changes in shape are temporary, and vary with the passive growth movements in the chain. See cell 5, figures 2, 3, 4, and 5.

As the number of cells in the droplet increases, the chains break up into smaller and smaller chains. Finally when multiplication ceases, chain formation is entirely lost, and the surface of the droplet becomes filled with organisms, which, however, still lie in only one plane and show no tendency to pile up.

Culture. *Mycobacterium phlei*, smooth strain, Lister Institute Strain number 54.

Medium. Glycerol synthetic medium solidified with 1 per cent agar.

Method. Cells from a forty-eight-hour culture on the solid medium were separated by shaking with glass beads, suspended in liquid medium and a loopful of this material added to a tube of the melted agar medium. A loopful of this inoculated agar was placed on the coverglass. The slides were incubated at room temperature and examined at intervals of an hour.

Data. Essentially the same phenomena were noted as in the case of Mycobacterium 5-3A. There was more curvatuve of the rods, sometimes almost corkscrew-shaped rods being seen, since the agar probably somewhat prevented the freedom of the passive movements due to growth pressure. Again, growth proceeded until it had covered the surface of the drop. The organisms are of many bizarre shapes at this stage due to the fact that they are fitted together with little unoccupied space. Examples of the shapes seen are shown in figure 6 which represents a three months' old growth. These polymorphic shapes are much more numerous in an agar medium than in a liquid medium, and may be due to the agar obstructing the displacement of one cell by the growth movements of another cell. The organisms develop at the surface of the agar, but one does not know whether they are growing in the film

of moisture on the agar surface or just beneath the agar surface. Also, the surface moisture may contain some agar.

Coccoid forms as noted by Wyckoff and Smithburn (1933) were very numerous in certain areas along the edge of the drop, at the time when multiplication had ceased. These forms were oval, the majority measuring about 1.2 micra by 0.8 micron, while the majority of the rods measured about 3 micra by 0.6 micron. Such coccoid forms also result from transferring rods to flat microdroplets of fresh medium (the flatness of the droplet is regulated by the amount of grease on the coverslip). As Wyckoff and Smithburn point out, the length of the organism depends upon the relation between the rate of division and the rate of growth. Since the moisture and food in the flat droplets are more rapidly exhausted than in more convex droplets, it is probable that these are the determining factors of the rate of growth in length. One or two rod forms placed in such microdroplets divide, the second generation cells then divide after very little growth in length, and so on. The subsequent divisions thus result in the formation of smaller and smaller cells. The droplet becomes filled with cocci, which never grow any longer even after multiplication has ceased.

Culture. *Mycobacterium tuberculosis*, avian type, smooth strain.

Medium. Glycerol synthetic medium solidified with 1 per cent agar.

Method. The same procedure was used as for *M. phlei*, except that a ten-day old growth on Dorset's egg medium was used as the source culture. The incubation temperature of the slides was 37°C.

Data. It was found that the microcultures of this organism in the young active stages of reproduction could not be distinguished from those of M. 5-3A or *M. phlei*. A lag phase of three or four days occurred, but the generation time in the logarithmic growth phase of five or six hours was comparable to that of the other two cultures studied. However, after division ceased, the cells grew in length. Since the organisms lay too close together for accurate microscopic study, some of these long cells were removed with micropipettes to microdroplets of liquid medium. Figure 7 shows cells from a six weeks' growth. The curved rods did not straighten out when released from the pressure of

the other cells, so that apparently the cell wall becomes more rigid upon aging. Some of the rods were 8 to 10 micra long, but no branching forms were seen. These cells divided by binary fission and grew as usual when transferred to fresh loopfuls of the agar medium.

Microcultures of the rough strains of the above three organisms have also been observed. Their colonies are all of the wavy strand variety as illustrated by Wyckoff (1934a) in plate 28, figure 62, for the rough variety of *M. chelonei*. A "snapping and slipping" growth, as postulated by Jensen (1934) for his subgenus A, was observed in the initial stages of growth of single cells of all these rough strains. The fate of individual cells cannot therefore be followed, and since piling up begins very soon, the shapes of individual cells cannot be distinguished after the first few divisions. Also, the cell outlines are always more hazy than those of the organisms of the smooth types. However, from observations on the cells during the first multiplications and on individual cells picked up by pipettes from the main growth mass in the later stages of growth, their morphology and manner of division differ in no way from that of the smooth cells.

Culture. The human tubercle bacillus, Kahn's single cell strain of H37.

Medium. Glycerol synthetic medium solidified with 1 per cent agar.

Data. The microcolonies developing are of the rough type and are burr-like in form. Again, as for the rough strains of the other species, individual organisms cannot be discerned, but those separated out from the colonies by pipettes are not branched and do not differ in other ways in their morphology from the other species observed.

Single cells of the human tubercle bacillus placed in microdroplets of liquid synthetic medium, as in Kahn's method, have never grown.

DISCUSSION

Our observations on the dividing cells of three species of Mycobacteria may be summarized by saying that under the conditions of the experiments these organisms reproduced by binary fission only, and that there was no evidence of branching or any other life cycle form. Coccoid forms resulted from the occurrence of division at a time when there was very little growth in length;

and relatively long rod forms resulted when division had ceased but growth continued. The limiting factor in growth in length appears to be the conditions of nourishment, but a reasonable conjecture as to the limiting factor for division is not manifest. Forms deviating in shape from the straight rod with parallel sides are found in young cultures. These are temporary shapes and change as the pressure from neighboring cells varies, since the cell wall is flexible. More widely aberrant forms, probably so-called "involution" forms, are numerous in cultures when growth and multiplication have ceased and result from the cells being packed closely together. The cell walls become rigid upon aging, so that the shape of the cell is retained even when the pressure from other cells is released.

Since our conditions were comparable, why did we not see the branching forms reported by other workers? If preparations such as those illustrated in the first five figures are examined magnified 440 times instead of 1425 times, pictures such as that of figure 8 are seen. It is obvious by comparing the same preparation under the two magnifications, that the appearance of branching at the lower magnification is due to the flexibility of the cells and the slipping out of line of certain cells due to growth pressure in a chain—some of the spaces between the cells being too small to be evident unless magnified at least 1000 times.

We therefore deduce that the branching noted by Miehe (1909) and Jensen (1934), using magnifications of about 750 times, and Wyckoff (1934a) using magnifications of about 350 times, was only apparent. This deduction is further substantiated by the fact that the pictures given by these authors are similar to our picture in figure 8.

According to our data, the pseudo-branching was seen only in microcultures of the smooth types of the three species observed. Wyckoff (1934a) found the branching rods most frequently in smooth cultures, but occasionally in rough ones; and Jensen (1934) does not discriminate, reporting branched forms in both plane and perrugose variants. We believe that these "branched" forms in the rough cultures were really smooth types—either the

rough source culture contained some smooth forms, or dissociation of the rough to the smooth occurred in the microcultures. The possibility of these two explanations has been experimentally verified. Single cell cultures of Mycobacterium 5–3A, smooth type, when dissociating into the rough type, will show the presence of all proportions of smooth, mucoid, and rough types (as shown by plating out) depending upon the stage of dissociation. In microcultures in mannitol synthetic broth, single cells of M. 5–3A, rough type, will dissociate into the smooth type, unless the cell is deposited on the coverglass near but not in a droplet. Some small amount of liquid remains around the cell of course, and when multiplication has started, a growth of the rough type will spread over onto the droplet. Cells of the smooth type of the avian tubercle bacillus, on the other hand, will dissociate into the rough type in droplets of glycerol synthetic broth. It is therefore necessary to start the smooth growth in an agar medium. We do not wish to go into a discussion of dissociation here, however, but merely to indicate its relation to morphology.

CONCLUSIONS

1. No evidence was obtained, from our observations on microcultures of three species of Mycobacteria, namely a saprophytic species isolated at the University of Wisconsin, the phlei bacillus, and the avian tubercle bacillus, that division occurs by any method other than binary fission.

2. Branching forms were not seen when magnifications of 1425 times were used. Pseudo-branched forms were seen in cultures of the smooth type magnified 440 times.

3. The cells are not uniform in size or shape. Coccoid and relatively long rod forms were seen to be functions of the ratio of the rate of division to the rate of growth in length. Bent rods, club-shaped rods, and such types result from the flexibility of the walls of young cells and the growth pressures from other cells.

4. No difference was apparent in the morphology or mode of division of one species from another.

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PLATE 1

FIG. 1. The growth from a single cell of Mycobacterium 5-3A after nineteen hours from the time of "planting" in a microdroplet of mannitol synthetic liquid medium. The numbers have been arbitrarily applied to the cells as a means of identification.

Figs. 1 through 7 are drawings made at magnifications of 1425 times.

FIG. 2. Same as figure 1 after twenty hours. Cells 1*a* and 1*b* are daughter cells of cell 1 in figure 1, etc.

FIG. 3. Same as figure 1 after twenty-one hours. Cells 7*aa*, 7*ab*, 7*ba*, and 7*bb* are daughter cells of daughter cells of cell 7 in figure 2.

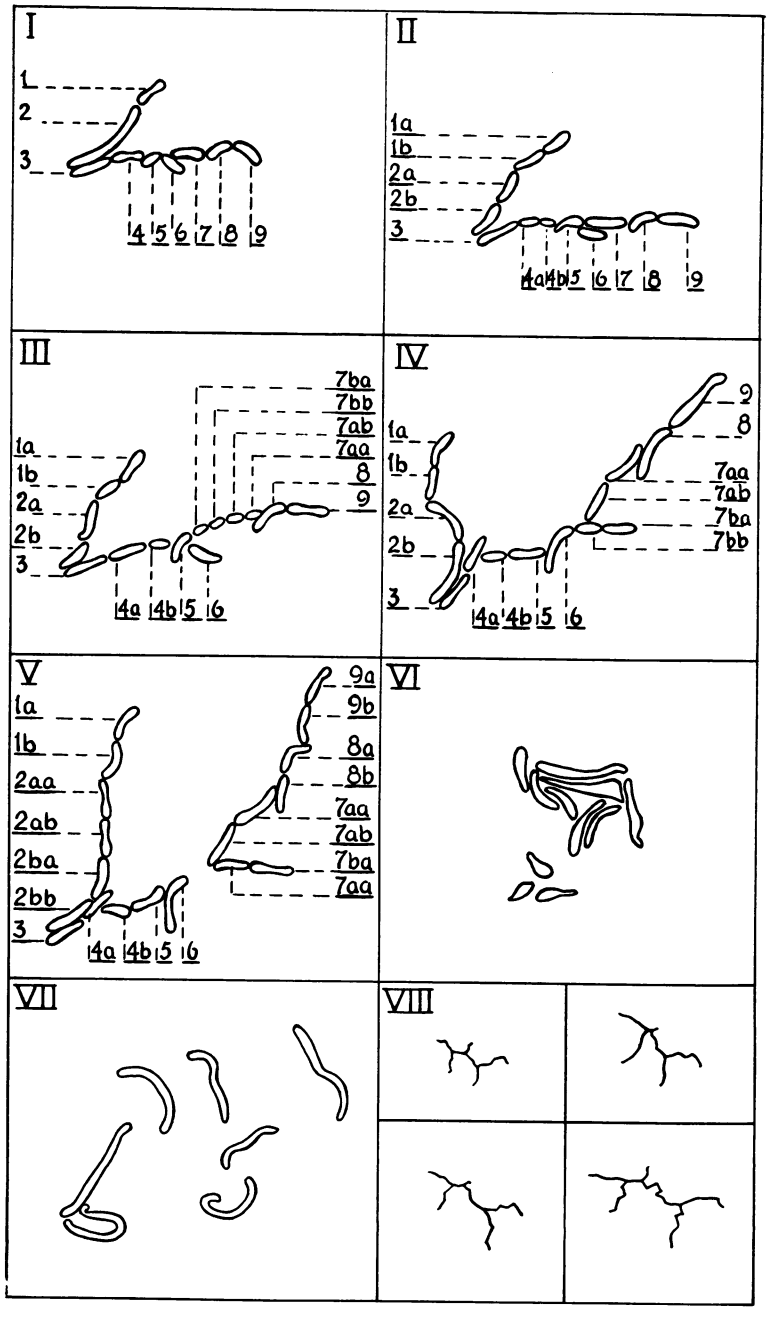
FIG. 4. Same as figure 1 after twenty-three hours.

FIG. 5. Same as figure 1 after twenty-five hours.

FIG. 6. Examples of cells of Mycobacterium phlei in a three months growth in a loopful of glycerol synthetic agar.

FIG. 7. Examples of cells of the avian tubercle bacilli in a six weeks growth in a loopful of glycerol synthetic agar.

FIG. 8. The growth from a single cell of Mycobacterium 5-3A after nineteen, twenty, twenty-one, and twenty-three hours respectively, in a microdroplet of mannitol synthetic liquid medium, as it appears when magnified 440 times.



(Janet McCarter and E. G. Hastings: Morphology of Mycobacteria)