ON THE NATURE OF THE GRANULES OF MYCOBACTERIUM AND THE IDENTITY OF THE BACTERIAL NUCLEUS

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In spite of the tremendous volume of literature on the bacterial nucleus, there is still no agreement among bacterial cytologists on what is a sure criterion of its identity. Witness the present status of the granules of Mycobacterium, variously considered as mitochondria (Mudd *et al.*, 1951), nuclei (Knaysi *et al.*, 1950; Knaysi, 1952), neither (Glauert and Brieger, 1955), or artifacts (Bisset, 1955). The present investigation attempts to find the reasons for these differences and demonstrates that these granules, regardless of their size or position, are real and are the cell nuclei.

MATERIALS AND METHODS

Most of the observations reported in this paper were made on *Mycobacterium thamnopheos*, although other organisms, particularly *Staphylococcus flavo-cyaneus*, were employed in certain phases of the investigation. The internal structure of both organisms is clearly revealed by simple staining with an acidified solution of a basic dye, such as methylene blue or thionine, regardless of the medium on which they are grown (Knaysi, 1951). Consequently, observations on the structure and behavior of intracellular granules in both organisms were made primarily on cells stained by methods A or C previously described (Knaysi, 1955*a*). In method A the cells are stained with an acidified solution of methylene blue (pH 3.1–3.2), and in method C they are stained first with a slightly acidified solution of thionine, then with a solution of methylene blue buffered with a mixture of K acid phthalate and HCl using the same pH as that in method A.

In all reported cases (with the exception of figure 2[1]), the organisms were grown at various temperatures on collodion films supported by various agar media. The media most commonly used were MITG/2: MI = meat infusion, T = 1 per cent tryptone, and G = 1 per cent glucose; and GA/5: G = 1 per cent glucose and A = 1 per cent Na acetate; the denominators indicate the dilution. Comparison of the same cells and structures after various consecutive treatments was made according to the procedure previously described (Knaysi, 1955*a*, 1957): for the most part between cells and structures stained by method A or C and by the HCl-Giemsa technique; or between cells and structures in microcultures grown on a collodion film supported by an agar medium containing 0.005 per cent neotetrazolium and the same cells and structures after staining by methods A, C, or HCl-Giemsa. Sometimes the acidfast or Gram reaction was included. Emphasis was placed on comparison of these methods to that of the HCl-Giemsa method not only to satisfy those who believe the significance of this technique, but also to test further the cytological value of this procedure.

Fixation with formol vapors was carried out by inverting the air-dry microculture, for a definite time, over the well of a culture slide containing a drop of commercial formalin. To fix the cells in the wet state with OsO_4 vapor, the floated microculture was picked up with a cover glass, thoroughly drained by placing it in a petri dish at an angle and absorbing the free water which collects at the border of the collodion film with blotting paper. The cover glass is then quickly inverted over the well of a culture slide containing a 0.25 per cent solution of OsO_4. After exposure for a definite time, the cover glass is removed and allowed to dry before further treatment, or sometimes immediately inverted over a drop of the staining solution, sealed, and examined.

For making a gram stain we used a 0.1 per cent solution of crystal violet for 1 min, rinsed the crystal violet with a solution containing 0.1 g of I_2 and 0.2 g of KI per 100 ml, and mordanted with a fresh portion of this solution for 1 min. Differentiation was made with 95 per cent ethanol for 20 sec but was not followed by a contrast stain. The reaction of acid fastness was carried out as

[1] The numbers and letters in parentheses after the figure number identify the negative in the author's library. Any figure without a scale has the same magnification as figure 1.

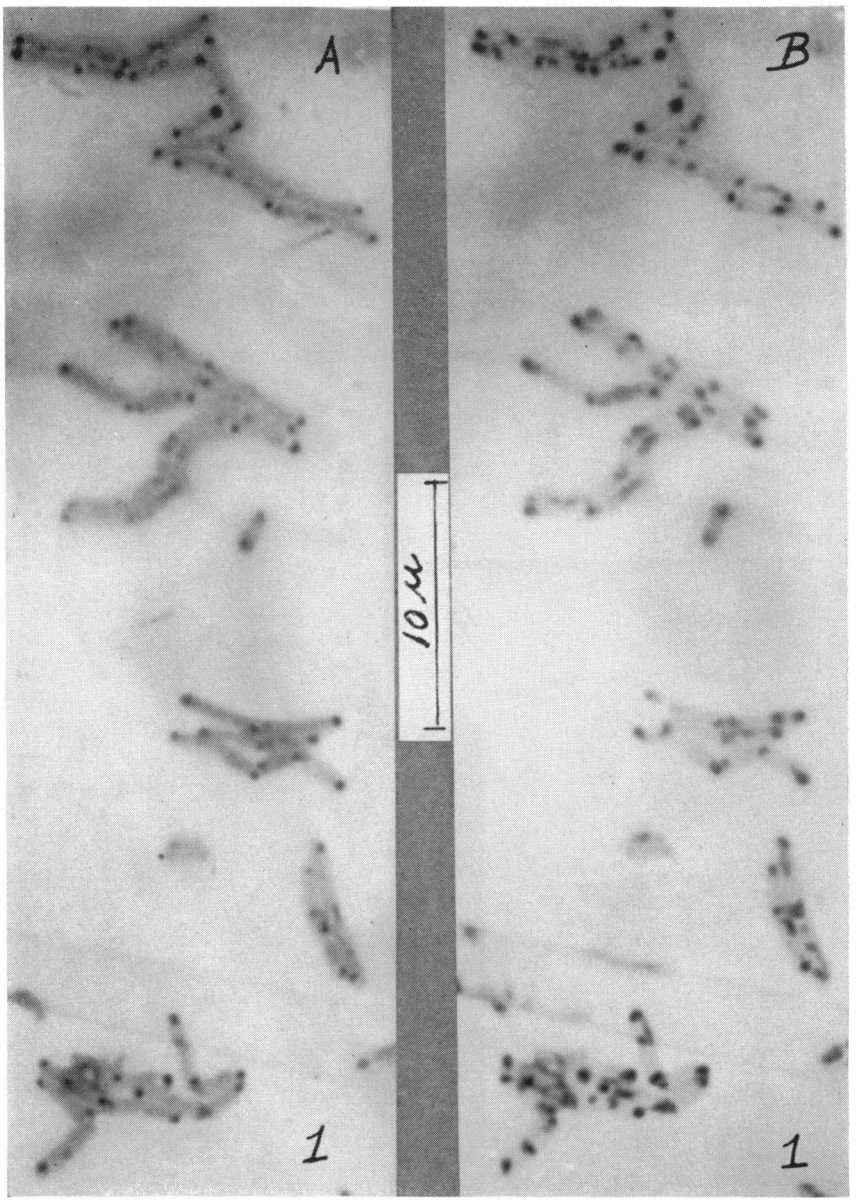


Figure 1A (55-D, 19). *Mycobacterium thamnopheos* microculture supported by MITG/2 agar; 7 hr old at 30 C; fixed wet with OsO_4 vapors for 1 min and mounted in an acid thionine solution.

B (55-D, 23). Same cells as in figure 1*A*, mounted in methylene blue solution of pH 3.15 after complete removal of free thionine. Note that in most cells the numbers and positions of the granules are the same as in the corresponding cells of figure 1*A*.

recently described (Knaysi, 1957), and is a slight modification of the usual Ziehl-Neelsen procedure. The fixed and air-dry preparation was covered with carbol fuchsin and heated from above with a Bunsen burner until mild steaming occurs. This is repeated 3 times. The preparation is then rinsed with running water and differentiated with 0.1 N HCl in 95 per cent ethanol. No contrast stain was used.

The acid treatment in the HCl-Giemsa procedure was carried out at various combinations of time and temperature. Following thorough rinsing

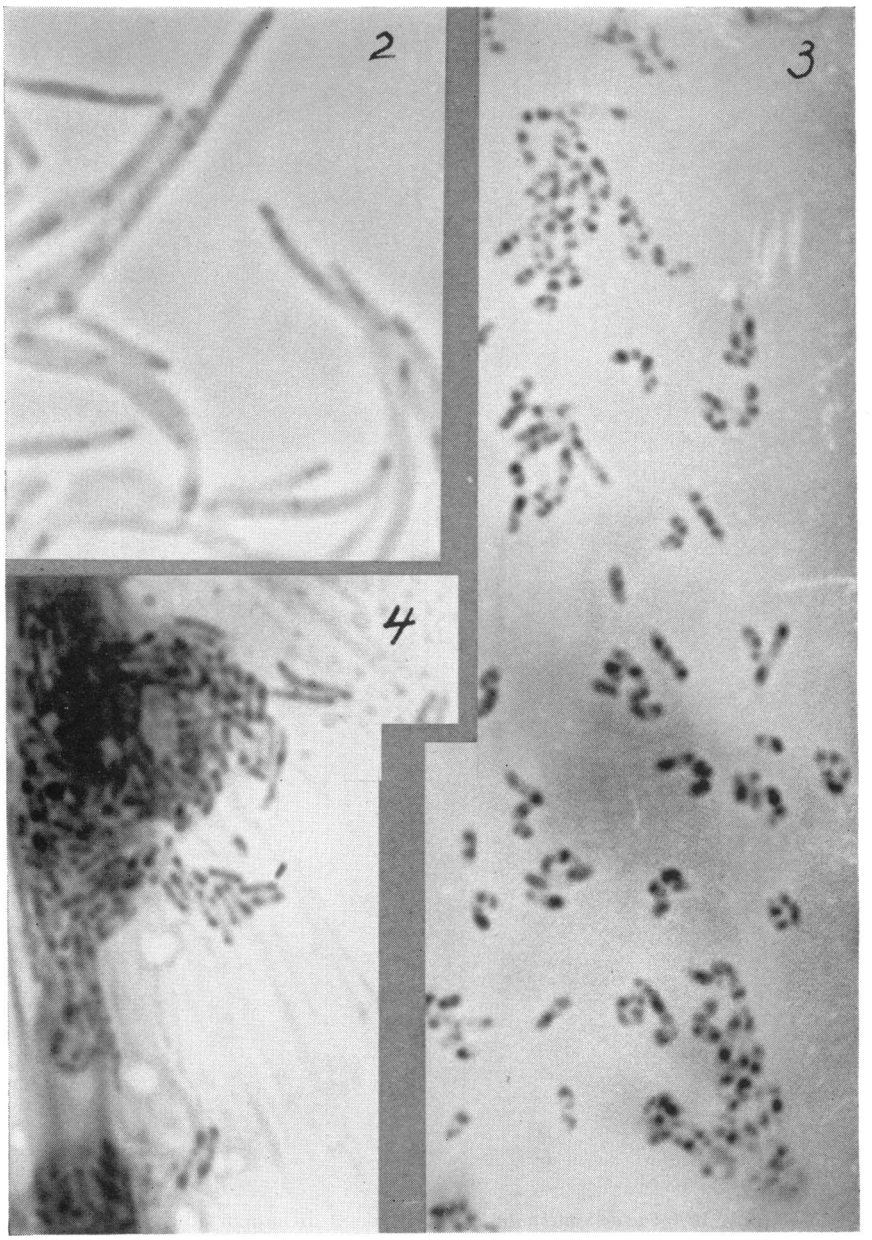
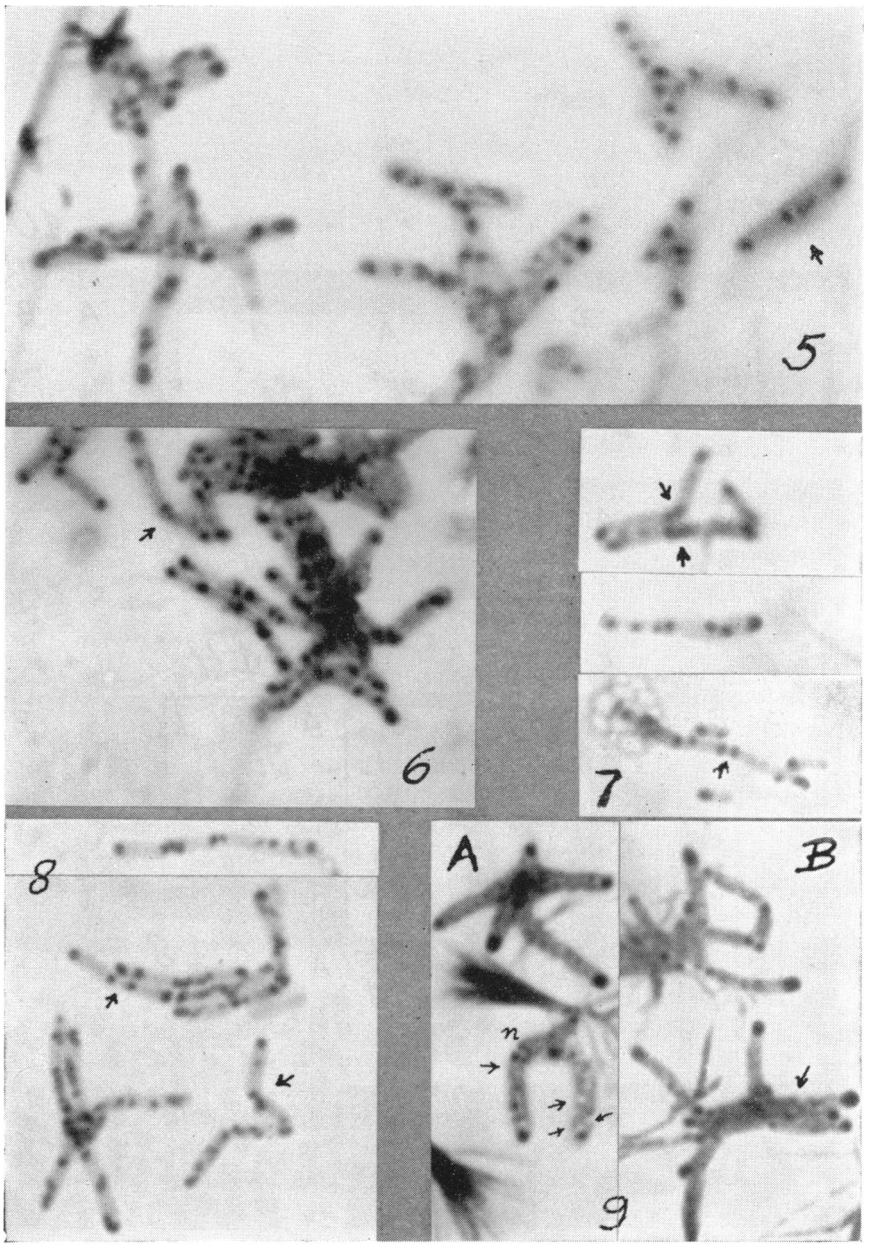


Figure 2 (51-AA, 23). *M. thamnopheos* living microculture grown on MITG/2 agar as described in Knaysi, 1952; age 31 hr and 45 min at 25–28 C; observed with the phase microscope in dark contrast.

Figure 3 (55-T, 1). *M. thamnopheos* microculture supported by MITG/2 agar containing 0.005 per cent neotetrazolium; 6 hr and 50 min old at 30 C; fixed for 2 min with formol vapors and mounted in water. Note that the stained granules vary considerably in their dimensions and may be found anywhere in the cell.

Figure 4 (55-Q, 17). *M. thamnopheos* microculture supported by MITG/2 agar containing 0.005 per cent neotetrazolium; 7 hr and 20 min old at 30 C; treated with N HCl at 58 C for 10 min and stained with Giemsa's solution. Note that the deeply stained granules, presumably nuclei, are mostly terminal.



Figures 5 to 8 (55-D, 21, 26, 13, and 15, resp.). *M. thamnopheos* microculture supported with MITG/2 agar; 7 hr and 30 min old at 30 C; fixed wet with OsO_4 vapors for 1 min and stained by the author's method C (Knaysi, 1955*a*). Note the many paired or dumbbell-like granules, sometimes at either side of the plane of division or cross wall (indicated by arrows) of the cell. Note also that the terminal granules, at this stage, do not differ much in size or appearance from granules in other positions.

Figure 9, *A* and *B* (55-A, 3 and 4, resp.). *M. thamnopheos* microculture supported by MITG/2 agar; 5 hr and 30 min old at 30 C; fixed with formol vapors and stained as for figures 5–8. Note the cell sap vacuoles (arrows) and in *A* dividing compound nucleus *n* at the plane of cell division.

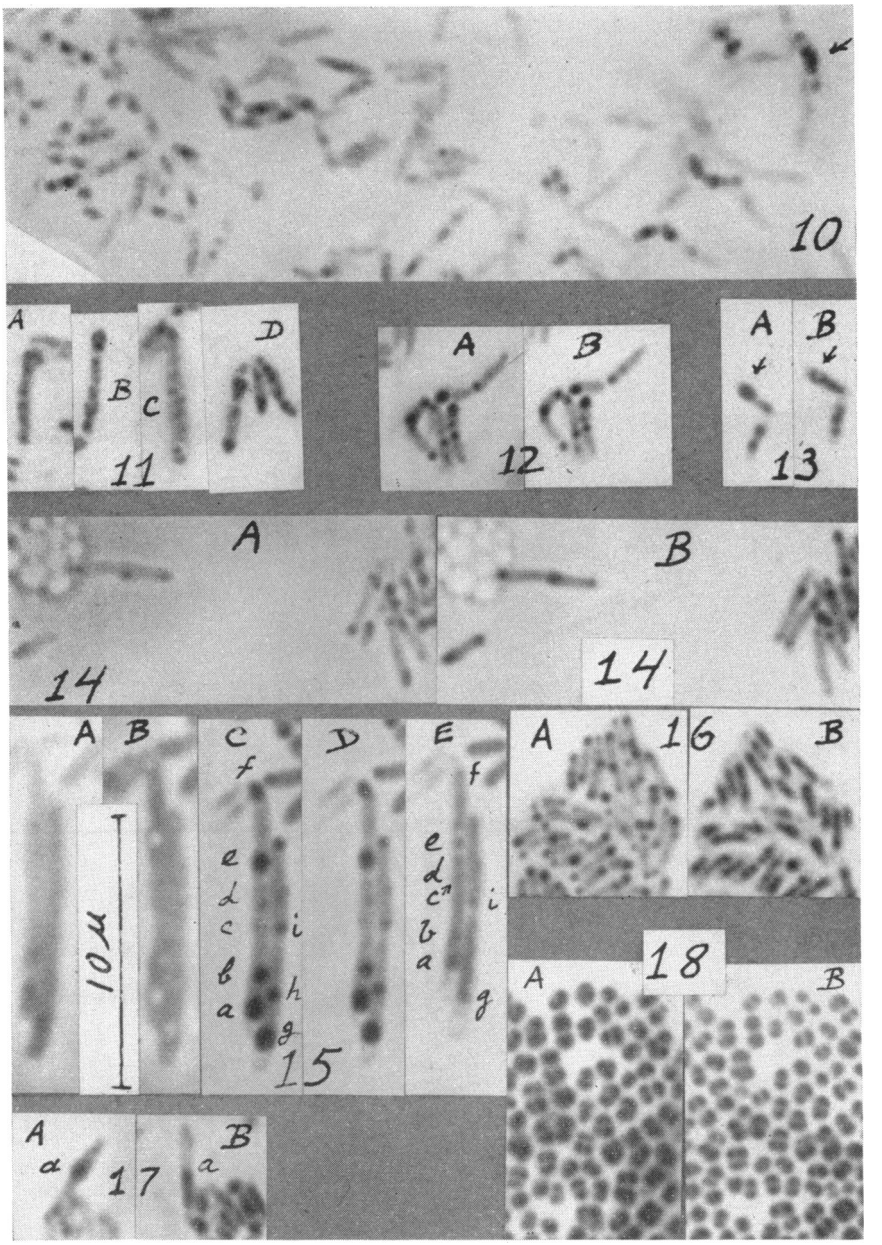


Figure 10 (55-C, 7). *M. thamnopheos* microculture supported by GA/5 agar; 18 hr and 30 min old at 30 C; fixed with formol vapors for 2 min and stained as for figures 5–8. Note that here the largest granule generally occupies a central position; smaller granules may or may not be present in the same cell. The granule indicated by an arrow has the structure of a compound nucleus.

Figure 11, A–D (55-C, 21, 32, 15, and 34, resp.). *M. thamnopheos* from microcultures supported by MITG/2 agar; age varying from 4 hr to 4 hr and 30 min at 30 C, fixed with formol vapors. *A*, *C*, and *D* stained as for figures 5–8; *B* stained by the author's method A (Knaysi, 1955*a*). Note the structure of the terminal granules, which is that of compound nuclei.

Figure 12A (56-G, 2). *M. thamnopheos* microculture supported by MITG/2 agar; 7 hr and 10 min old at 30 C; fixed with 95 per cent ethanol for 10 min and stained as for figure 11*B*.

B (56-G, 3). The same cells as those of *A* stained by a Gram method (*vide* text). Note that the gram positive granules seen here and the granules seen in *A* are the same.

with running water, the preparation was stained for 10 min with Giemsa's solution diluted 5 times, mounted in water, and observed.

The photomicrographs were taken on 36 mm, microfile film with a 90X apochromatic objective, N.A. = 1.30, and a special attachment to the microscope. Magnification on the film was 450 ×.

RESULTS

Reality of the granules. The granules are visible in the living cell (figure 2 and Knaysi, 1952). They are demonstrable in cells that have been subjected only to quick drying and, with or without short exposure to gaseous formaldehyde or to alcohol, have been mounted in dilute thionine or methylene blue solution (figures 1, 15*C*, 16*A*, 17*A*). They are demonstrable with the electron microscope (Knaysi *et al.* 1950). Barring philosophical skepticism, their existence as natural components of the cell is as well founded as the existence of any intracellular body.

Size, number, and position. The present observations are in complete agreement with those previously made (Knaysi, 1952) on the same organism in the living state with the phase microscope, and with those of Knaysi *et al.* (1950) on *Mycobacterium tuberculosis.* In actively growing cultures the granules are generally small or of moderate size and may be distributed throughout the cells with a tendency to occupy terminal and median positions (figures 1, 5–8). The terminal and median granules in particular grow in size as the culture matures, and this growth in size is usually accompanied, except in filamentous growth (figure 15), by a reduction in the number of granules per cell. In a mature culture (figures 10, 16*A*), the cell may contain two terminal granules with or without a third in a median position. When there are three, the largest granule may be terminal or median. Large, terminal or median granules may be found in very young cultures. These may be in slowly growing cells of the

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Figure 13A (56-B, 40). *M. thamnopheos* microculture supported by MITG/2 agar; 24 hr old at 30 C; fixed for 35 min with 95 per cent ethanol and stained as in figure 11*B*.

B (56-C, 10). Same cells as in *A* treated with N HCl at 58 C for 10 min and stained with Giemsa's solution. Note that the terminal granule indicated by the arrow lost its content upon treatment with the acid, leaving a deeply staining, peripheral, horseshoe-like, presumably chromatin containing body, just as would be expected from a compound nucleus.

Figure 14A (55-P, 18). *M. thamnopheos* microculture supported by MITG/2 agar containing 0.005 per cent neotetrazolium; 6 hr and 10 min old at 30 C; fixed with formol vapors for 2 min and mounted in water.

B (55-P, 22). The same cells as those of *A* stained as for figure 11*B*. Note that the granules which take up reduced neotetrazolium and those which stain by the author's method *A* are the same.

Figure 15, A–E (56-J, 3, 5, 9, 12, and 14, resp.). *M. thamnopheos* microculture supported by MITG/2 agar; 29 hr and 30 min old at 30 C; fixed with formol vapors for 2 min. *A* mounted in water and observed in dark contrast with the phase microscope. *B*, the same cells as those of *A* stained by the author's method A and observed in dark contrast with the phase microscope. Note that the granules, which were dark before staining, now appear bright in the center with a dark rim. *C*, the same cells and treatment as in *B*, observed in bright field. *D*, the same cells as in *A* to *C* stained by the Ziehl-Neelsen method. *E*, the same cells as in *A* to *D* treated with N HCl at 60 C for 10 min and stained with Giemsa's solution. Note that granules *a–i* of *C* and *D* were not completely removed by the treatment with acid, but that some (e.g. *a*, *b*, and *g*) were pulled toward the opposite end of the cell by the shrunken cytoplasm. The original positions of these two granules as well as those of *g* and *h* are still outlined and appear as vacuoles.

Figure 16A (56-B, 39). *M. thamnopheos* microculture supported by MITG/2 agar; 24 hr old at 30 C. Fixed 35 min with 95 per cent ethanol and stained as for figure 11*B*.

B (56-C, 7). The same cells as those of *A* treated with N HCl at 58 C for 10 min and stained with Giemsa's solution. Note that the cytoplasm has shrunk, from both ends toward the median part of the cell, into a stainable mass that has obscured the encosed nuclei.

Figure 17A (56-B, 34). *M. thamnopheos* microculture and treatment as for figure 16*A*.

B (56-B, 37). The same cells as those of *A*; same treatment as for figure 16*B*. Note a case of excessive shrinkage of the protoplasm of cell *a*.

Figure 18A (56-E, 12). *Staphylococcus flavo-cyaneus* microculture supported by MITG/2 agar; 17 hr and 25 min old at 30 C; fixed with 95 per cent ethanol for 10 min and stained as in figure 11*B*.

B (56-E, 13). Same cells as those of *A* treated with N HCl at 60 C for 10 min and stained with Giemsa's solution. Note that both methods show the same internal granules (nuclei).

inoculum, and some are probably in the process of dividing (figures 9*A*, 11*C*, *D*). It must be emphasized that this description is only general and that considerable departure from this picture may be found in individual cells.

Staining properties. The granules stain deeply with neutral dyes, and with basic dyes even at a very low pH. They may stain red, regardless of size or position, in cells grown on a medium containing neotetrazolium (figure 3). They are strongly gram positive (figure 12*B*) and acidfast (figure 15*D*; also *vide* Knaysi, 1957). When stained with methylene blue, the small bodies appear dark blue with a reddish tone at the periphery. The large and intermediate granules show a dark blue periphery or peripheral bodies and red central ground material. This appearance is exactly the same as that observed (Knaysi, 1955*a*) in the compound nuclei of *Bacillus cereus*.

Structure and behavior. Present observations on the structure of the granules are in complete agreement with those previously made (Knaysi, 1952) on living cells. Furthermore, the structure of the small granules resembles that of the primary nuclei (figures 1*B*, 5, 6), and the structure of the intermediate and large granules that of the compound nuclei (figures 9*A*, 11, 13) as previously described (Knaysi, 1955*a*). In the cell indicated by the arrow in figure 13 the nucleoplasm with its chromotropic content was removed from the large, terminal granule by treatment with acid, leaving a peripheral, chromatin-like body—strong evidence that the granule is a compound nucleus.

Division and fusion of the granules were demonstrated in the living cells of this organism (Knaysi, 1952). Present observations on form, organization, and relative position of the granules, taking into consideration the state of the cells with respect to vegetative reproduction (figures 1*B*, 5–8) and the change in the size of the granules and in their number per cell (figures 10, 16*A*), are consistent with the division and fusion of these structures. In the numerous cases in which we observed apparent division, we found no evidence of mitosis.

Comparison of methods. Method A *vs* growth on neotetrazolium containing media:—In making this comparison we soon realized that some of the cells grown to what we consider to be the proper stage on the neotetrazolium media tend to stain uniformly with our method A. This, we believe, is due to autolysis, since cells in which all the granules are stained by reduced neotetrazolium are dead. However, many other cells continue to stain normally, and these clearly show that the same granules are stained by both methods (figure 14).

(2) Method A *vs* HCl-Giemsa staining. Our first attempts to make this comparison were highly discouraging as altogether different pictures were presented by the methods. Cells stained by method A appeared true to life (figures 2, 19*A*), whereas those stained by the HCl-Giemsa technique appeared highly disorganized (figures 16*B*, 17*B*, 19*B*). Wide variation of time and temperature of acid treatment failed to change the picture materially. A study of many experiments similar to those illustrated in figures 15–17 and 19 indicated that, although certain cells here and there may still show an internal structure roughly similar to their true structure (figure 13 and cells *f* and *g* of figure 19), the structural alteration observed in the great majority of the cells appears to be due to shrinkage caused by the acid treatment. A certain type of cells, often containing one granule or two terminal granules, shrinks from both ends toward the middle, usually forming one large, deeply staining mass in the median part of the cell. This mass contains the granules or residues of the granules originally present in the cell. Terminal granules may often be distinguished as darker areas at their corresponding ends of the large mass (figure 16). Cells with 2 terminal and 1 central granule may shrink into 1 or 2 masses, probably depending on whether the cytoplasm is continuous or divided. In other cells, shrinkage takes place chiefly from one end of the cell toward the other and, on superficial examination, often gives the impression that a terminal granule originally present in the cell was removed by the acid treatment, leaving a vacuole (figures 15*C*, *E*, and 19). Careful examination shows, almost invariably, that the terminal granule, or its residue, is still visible at the corresponding end of the shrunken protoplasm (granules *a* and *g* in figure 15*C*, *E*, and cells *a–d*, *f*, and, *g* of figure 19). The granule has not disappeared, but has been merely displaced. Other granules that may be present in the same cell keep their respective positions (granules *b–f* of figure 15*C*, *E*, and the lower terminal granules of cells *f* and *g* in figure 19). An extreme case of this asymmetric type of shrinkage is shown in figure 17. Small granules within the cytoplasm may undergo a

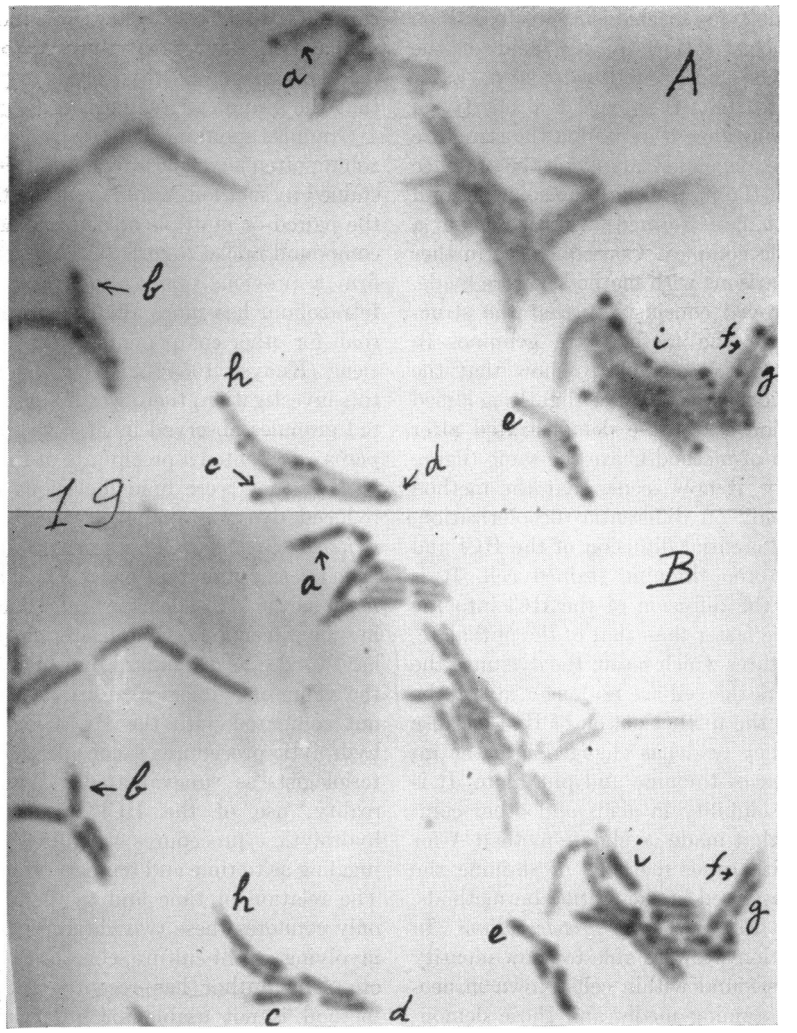

Figure 19A (55-W, 25). *M. thamnopheos* microculture supported by MITG/2 agar, 6 hr old at 30 C; fixed with formol vapors for 2 min and stained as in figure 11*B*.

B (55-W, 32). The same cells as those of *A* treated with N HCl at 60 C for 10 min and stained with Giemsa's solution. Note that the terminal granules of cells *a–i* are still clearly visible at the corresponding ends of the shrunken cytoplasms.

reduction in ability to be stained but they are not replaced by colorless vacuoles. Under certain conditions (figure 4) shrinkage may be greatly reduced, and one can see that the large terminal and central granules, contrary to recent claims, are clearly stainable by this method.

Another change brought about by the acid treatment is an increase in the ability to be stained of the cytoplasm, a reversal of the role it is supposed to fulfill. This is usually explained by assuming that the acid treatment was too strong. Radical changes in time and temperature, however, did not remedy the situation.

Correspondence between the results of method A and that of the HCl-Giemsa when both are usable is shown in figure 18.

DISCUSSION AND CONCLUSIONS

Mechanism of method C. When method C was developed (Knaysi, 1955*a*), we were able to convince ourselves, whenever method A was usable, that both methods gave identical results. At first

we assumed that its mechanism was based on differential decolorization by the acid of the thionine stained nucleus and cytoplasm. Later study revealed that the special property of method C, for instance in revealing the structure of the forespore, depended largely on the presence of phthalate in the methylene blue solution, and that the phthalate formed with thionine a highly insoluble complex. Consequently, further exacting comparisons with method A were made. The results proved conclusively that the structures shown by the method are genuine. In Mycobacterium, it is possible to show that the structures demonstrated by the slightly acidified thionine solution and those demonstrated after the completion of method C are the same (figure 1). Accordingly, it now seems that the method depends not only on differential decolorization, but also on differential diffusion of the HCl and phthalate into the thionine stained cell. It is probable that the diffusion of the HCl into the cells takes place faster than that of the phthalate, and that structures which retain the dye until the phthalate enters the cell are no longer able to be decolorized by the further action of HCl because the thionine they held has changed into the insoluble complex of thionine and phthalate. It is chiefly this insolubility in acids and other common reagents that made us choose method A for comparison with other methods of staining the granules, as described in the section on methods.

Spontaneous staining with neotetrazolium. In a previous section we were able to show identity of the granules found within cells grown in neotetrazolium-containing media and those demonstrable by method A (figure 14). Technically, this identity is easy to prove by our method provided one employs cells of the right stage of development. Cells from very young cultures may not show red granules, or only some of their granules may be stained red. Cultures more than two days old may consist almost entirely of colorless cells, i. e., of cells that can grow in the presence of neotetrazolium unhindered. Assuming that these cells still have the power of reducing neotetrazolium to the red stage, their lack of color probably means that they have become impermeable to neotetrazolium, or that they have preserved the impermeability of ancestral cells. Cells in which all the granules are stained are no longer viable. The red granules are not easily decolorized by acid or alcohol and may retain their color after acid treatment as in the Feulgen technique. Because of this, cells grown on neotetrazolium media should never be subjected to the Feulgen reaction unless a posthydrolysis inspection shows that the granules have been decolorized.

Granules spontaneously stained with neotetrazolium often have a less regular outline than those stained by method A, and are more likely to show the paired or multiple structure characteristic of compound nuclei (figure 3). This appears to confirm a previous observation that reduced neotetrazolium has more affinity for the chromatin than for other components of the compound nucleus (Knaysi, 1955*a*). Under the conditions of this investigation, there was no evidence that the red granules observed in *Mycobacterium thamnopheos* represented a precipitate of the reduced dye or that they were lipid inclusions in which the reduced dye was dissolved.

Remarks on the HCl-Giemsa stain. Perhaps there is no nuclear stain that has received as much publicity as the HCl-Giemsa stain. Indeed, it is held in such esteem by certain bacteriologists that they have used it as a standard by which they measure the value of cytological research. Any technique not compared with the HCl-Giemsa or similar hydrolytic procedures is considered by these bacteriologists as "unevaluated" (Widra, 1956). In reality, use of the HCl-Giemsa and similar hydrolytic procedures requires considerable juggling as to time and temperature of hydrolysis. The relation of time and temperature here not only connotes these two variables; it is complex, involving age of culture, composition of medium, etc. The author has recently shown that the method is not usable for observing the structure of the forespore (Knaysi, 1955*b*). The present work shows convincingly how destructive it can be in Mycobacterium. We hope that these instances will discourage indiscriminate use of hydrolytic methods in general.

It was shown in a previous section that the damage done by the acid treatment is due chiefly to shrinkage. However, one wonders why this treatment does not cause similar damage in the cells of some other bacteria, such as those of the genera *Bacillus* and *Escherichia*. The answer to this, we believe, is that the cells of mycobacteria are permeated by cell-sap vacuoles. Enhancement of the ability to be stained of the cytoplasm by the acid treatment may well be due to inability of liberated nucleic acid, or nucleic acid fragments, to escape from the cytoplasm.

The intracellular granules of *Mycobacterium*

thamnopheos are not just of two sizes and positions. They present a gradation of sizes and may be found, regardless of their size, anywhere in the cell, most frequently near the ends or at the middle. During the period of rapid growth most granules are relatively small, becoming larger and fewer as the culture matures. The enlarged granules are also most commonly located near the ends and the middle of the cell. The structure and staining properties of intermediate and large granules is similar to that of the compound nucleus recently described in *Bacillus cereus* (Knaysi, 1955*a*). Simultaneous increase in the size of the granules, particularly the terminal and central ones, and decrease in their number is in harmony with the previous observation (Knaysi, 1952) that enlargement of the granules is due chiefly to association, i. e., fusion without loss of identity.

Regardless of size, the granules spontaneously stain red when the cells are grown on a medium containing neotetrazolium, and are strongly acidfast and gram positive. These same granules are also demonstrable with our methods A and C.

The HCl-Giemsa method is highly destructive to the cells of *Mycobacterium thamnopheos*, but the resulting disorganization follows certain patterns which may be studied by comparison with various, less destructive procedures such as method A. Such studies helped to prove that the large granules consist of superficially located, hydrolysis-resistant bodies having the staining properties of chromatin, and of a homogeneous, chromotropic, strongly basophilic ground material that is removed by hydrolysis (figure 13); consequently, that the intermediate and large granules of *Mycobacterium thamnopheos* are compound nuclei. There is considerable evidence for the division of these and the smaller granules (figures 1–9), i. e. of the compound and primary nuclei, but there was no indication of division by mitosis. Study of the disorganization wrought by acid hydrolysis suggests that it is due to vacuolization of the cells of mycobacteria first observed nearly 30 years ago (Knaysi, 1929), beautifully demonstrated with the electron microscope (Knaysi *et al.*, 1950), and now for the first time recorded photographically with the light microscope (figure 9). Cells that did not exhibit much disorganization (figure 13) may have a small number of vacuoles, which is suggested by their gross morphology as learned from previous studies.

SUMMARY

Observations of the same cells of *Mycobacterium thamnopheos* after each of various consecutive treatments showed that the granules demonstrated by method A (Knaysi, 1955*a*), regardless of their size, are strongly acidfast and gram positive and are identical with the granules which spontaneously stain red when the cells are grown in neotetrazolium containing media. Study of the disorganization observed when the cells are subjected to the HCl-Giemsa technique led to the conclusion that the small granules are the primary nuclei and the intermediate and large ones the compound nuclei previously described (Knaysi, 1955*a*) in *Bacillus cereus*. The destructive effect of acid hydrolysis on the cellular organization of Mycobacterium is attributed to the presence of vacuoles in the cells of this organism.

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