THE CYTOLOGY OF AN AVIAN STRAIN OF MYCOBACTERIUM TUBERCULOSIS STUDIED WITH THE ELECTRON AND LIGHT MICROSCOPES

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The early literature on the cytology of *Mycobacterium tuberculosis* was reviewed by Knaysi (1929). Among the reports not reviewed by him are those of Feinberg (1900), Nakanishi (1901), Minder (1916), Kirchensteins (1922), and Petit (1926). Feinberg observed a red nucleus and a blue cytoplasm in cells stained by a modified Romanowski's solution. Nakanishi demonstrated by postvital staining with methylene blue a round body located near the middle of the cell; sometimes there was a constricted body or there were two round bodies. Minder distinguished between Much's granules and polar bodies, although the latter were demonstrable by Much's method of staining. He stated that the granules were neither spores nor decomposition products. Kirchensteins, using a complicated method of staining with basic fuchsin, concluded that a nuclear apparatus existed consisting of several granules united by fine filaments. Petit did not attribute to the granules a nuclear nature.

Knaysi (1929) studied a strain, originally of the human type, which had lost its pathogenicity; his observations were summarized as follows:

> The young cell of *Mycobacterium tuberculosis* consists of a membrane presenting thickened areas and granular appendages on its internal surface; this membrane surrounds a very dense, deeply staining cytoplasm permeated by a vacuolar system and enclosing dense, round or oval hyperchromatic granules. The membrane and the granules seem to be made up of similar substances staining metachromatically with dilute, old methylene blue solutions and taking up iodine and the fat dyes to a great extent. This substance is not removed by boiling in water for one hour, nor by 5 per cent sodium hydroxide, 5 per cent sulfuric acid, glacial acetic acid or chloroform, at the end of a week. In old cells, the membrane increases in thickness and undergoes, together with the granules, gradual degeneration. The cell divides by drawing back of the protoplasm and the formation of two closing membranes, without constriction of the mother cell at the zone of division. The granules may divide, but they do not seem to be associated constantly with cell division. The present investigations do not substantiate the assumption of a wax or fat sheath around the cell of the tubercle bacillus, nor of wax or fat granules inside of the cell.

"Wax or fat granules" means granules consisting solely of these substances. A later study of cell division (Knaysi, 1941) showed that the process is fundamentally the same in *Mycobacterium tuberculosis* as in other bacteria.

Roman (1930) concluded that the Koch bacillus has neither a typical nor a spiral nucleus and stated:

The nuclear apparatus of this organism has not yet been demonstrated, perhaps because of its extremely small size. It is possible that the chromatin is disseminated in the cytoplasm as extremely fine particles, or that the nuclear substance is diffuse in the cytoplasm.

Hollande and Hollande (1932) described the structure of *Mycobacterium tuberculosis* in considerable detail: Young cells stain uniformly blue with methylene blue eosinate; therefore, they contain a dense, nonvacuolated, strongly basophilic cytoplasm. Old cells stain red with the same dye; therefore, they are mostly free of cytoplasm. Both types contain internal granules that stain violet blue with methylene blue eosinate and divide, sometimes unequally; these are the metachromatinosomes. Controlled differentiation with running water reveals the existence, in each metachromatinosome, of a minute, black-blue body, the nucleosome, which always divides equationally. The nucleosome is the nucleus of the cell. In young cells the membrane is barely visible; old cells have easily demonstrable, acid-fast membranes and contain strongly acid-fast granules. Acid-fast granules are formed, apparently by absorption of the cytoplasm into each metachromatinosome and the formation around it of a coat which stains red with methylene blue eosinate. According to Hollande and Hollande, an acid-fast granule is capable of germination, a phenomenon that has been reported by a number of investigators. Lindegren and Mellon (1932) claimed to have observed, in a hanging drop, a cytological process resembling the meiosis of fungi and higher forms. Lewis (1941), in his excellent review, reported the findings of Grimme (1902), Meyer (1912), Knaysi (1929), and Hartman (1940) that the granules of *Mycobacterium* stain with fat dyes and pointed out that, in contrast with the other investigators, Knaysi did not consider these granules to be lipoid inclusions.

After the development of the electron microscope, *Mycobacterium tuberculosis* was one of the first microorganisms to be examined. Mudd, Polevitzky, and Anderson (1942) observed a very delicate cell wall and dense, intracytoplasmic granules 70 to 230 mμ in diameter. Lurie, Knaysi, and Mudd (1942) examined a number of strains of the human, bovine, and avian varieties. The cytoplasm appeared homogeneously semitransparent, usually contained one or two opaque granules, and was surrounded by a thin cell wall. Rosenblatt, Fullam, and Gessler (1942) studied several species of *Mycobacterium* including bovine and avian varieties of *M. tuberculosis*, *M. phlei*, *M. leprae*, *M. marinum*, and *M. smegmatis*. The cells of the avian variety of *M. tuberculosis*

were usually rod-shaped and averaged about 1.0 μ in length and 0.3 μ in width. . . . There were occasionally found much longer bacilli, one of which measured 6.0 $\times$ 0.7 μ. A surrounding membrane was present which often contained granules. The nuclear material was either confined to the periphery or distributed throughout the long axis of the bacillus. The cytoplasm appeared granular.

The other cultures studied had a generally similar structure. The authors admit that their labeling of the internal granules as nuclei was arbitrary. Scanga (1948) published eight electron micrographs of *Mycobacterium friburgensis*, in one of which there is indication of two types of intracytoplasmic bodies: one is highly

opaque and is probably homologous to the dense bodies observed by the other investigators; the other is semitransparent and surrounded by a fine, opaque line.

ORGANISM

The organism studied belongs to the avian variety of *Mycobacterium tuberculosis* and is known as the Sheard strain; it was obtained from the Trudeau Sanitarium, Saranac Lake, New York, for a study on the relation of virulence to environment. A casual observation with the electron microscope convinced us that it was highly desirable to make a thorough study of its cytology both with the electron and with the light microscopes. The cytoplasm is highly transparent to the electron beam and has a very slight affinity for dyes, characteristics that should allow observations of the internal structure without the necessity of treatment, physiological or chemical, to remove the ribonucleic acid.

MEDIA AND METHODS

Our early studies with the electron microscope were made on cells grown in the liquid, synthetic medium described by Dubos *et al.* (1946). The cells were centrifuged out, washed with distilled water, deposited on dry collodion films supported by disks of copper screen, air-dried, and examined. It was found, however, that in submerged cultures the organism produced a considerable amount of dense slime, highly opaque to the electron beam; the cells appeared as transparent vacuoles inside the dark mass of slime (figure 1), and slime particles or filaments located at the surface of the cell often gave the impression of being internal or peripheral cellular structures (figure 2). It was found, however, that this troublesome slime was absent when the cells were grown on a collodion film deposited on the surface of an agar medium according to the technique of Hillier, Knaysi, and Baker (1948). In these collodion microcultures, the organism grows for a number of days mostly in a monocellular layer, and the cell structure can be clearly seen. This technique was, therefore, exclusively used in subsequent work with the electron microscope and was adapted for use with the light microscope. The adaptation consisted in picking the floated culture with a 22-by-40-mm cover glass, instead of a strip of copper screen, and the preparation was placed at a steep angle with one of the sides of the cover glass resting on a blotting or filter paper until air-dry. We found it convenient to rest the preparation against the inner side of an open petri dish the bottom of which was covered with a disk of filter paper. When such a preparation is air-dry, the collodion film adheres tenaciously to the surface of the cover glass, and the preparation can be fixed, stained, rinsed, etc., as if the cells were deposited directly on the glass. The collodion film remains practically unstained and does not interfere when the cells are observed.

The medium used most was that of Dubos and his co-workers (1946), to which was added 20 g of agar, but no albumen, per liter. However, identical results were obtained when the concentration of some of the mineral constituents of this medium was changed, or when the collodion culture was grown on beef

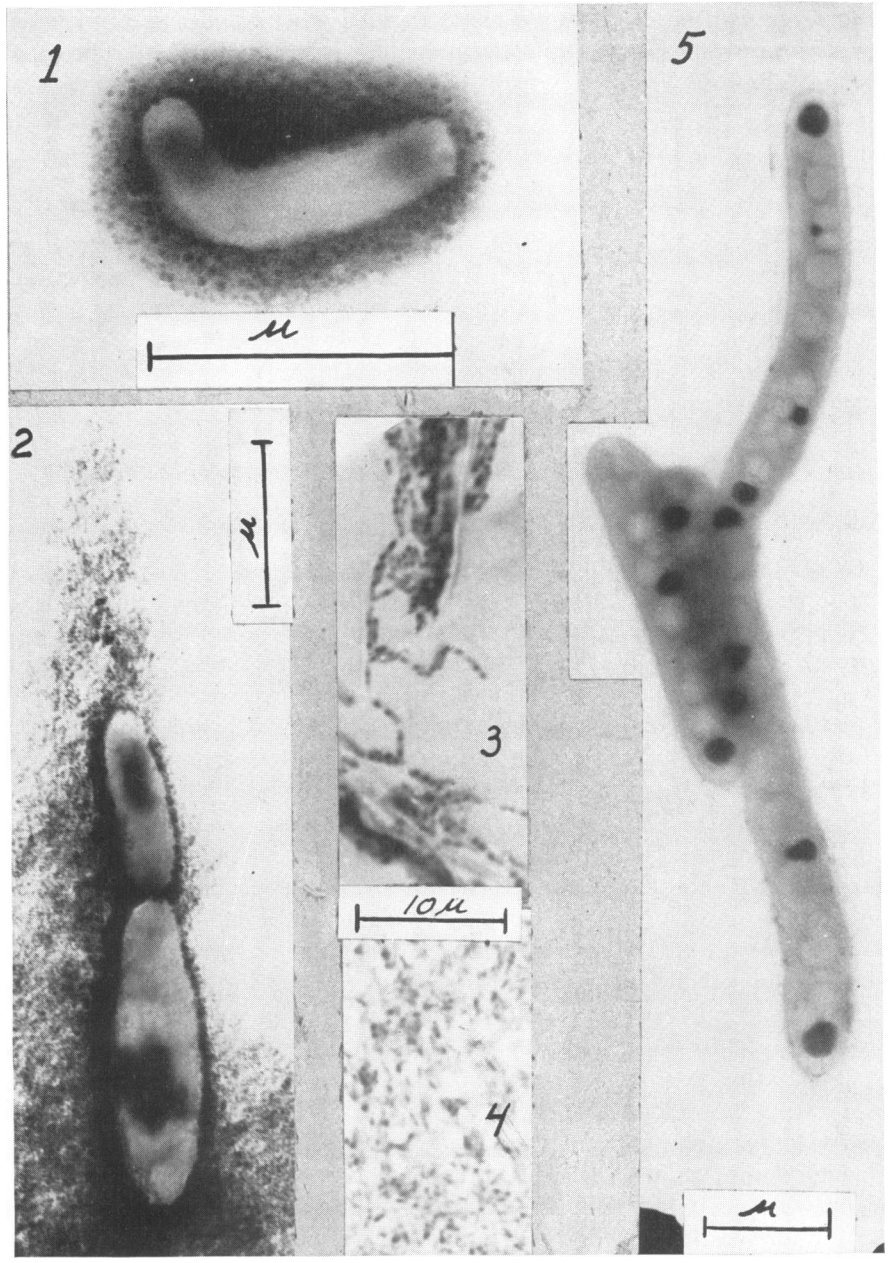

Figure 1. From a test tube culture in a liquid medium that differs from that of Dubos *et al.* in the concentration of the mineral components. The culture was 12 days old, two of which were at 37 C and the rest at room temperature. The cells were centrifuged out and washed with distilled water, and a droplet of the water suspension was dried on the collodion film. Note the thickness, density, and structure of the slime layer and the semitransparency of the cell.

Figure 2. From a test tube culture in the liquid medium of Dubos *et al.* The culture was

extract, tryptone agar. The cultures were incubated at 36 to 37 C with the petri dishes closed, and the atmosphere of the incubator was made humid by means of a tray of distilled water. The age of the cultures examined varied from less than a day to about two weeks.

Fixation and staining. Except in the phase involving the use of desoxyribonuclease, the air-dried preparation was fixed for 30 minutes in 95 per cent alcohol. This method of fixation gave pictures that agreed well with electron micrographs and with material stained without previous fixation. In addition, fixation with alcohol had the advantage of increasing accessibility of the cellular structures to the dye by dissolving lipids that tend to hinder penetration of the dye. Indeed, we are convinced that the use of saturated alcoholic solutions of dyes in the preparation of a number of well-known stains was not merely a convenient way of adding the dye, but that the small amount of alcohol added facilitated and improved stainability.

In general, the structure and behavior of the granules were followed in preparations stained for 10 minutes with Giemsa's solution diluted five times with distilled water. Figure 3 shows that it was optional for us to use any number of dyes for that purpose. Since the cytoplasm of this organism remains practically unstained unless special mordants are used, it was not necessary to subject the cells to hydrolysis with acid or any other treatment to remove stainable substances from the cytoplasm; nevertheless, figure 4 shows that hydrolysis does not alter the general picture, althought it somewhat reduces its clarity.

Lipid was demonstrated with Sudan III. The collodion culture is picked with a cover glass and the preparation allowed to drain; water droplets that do not run off are removed with a strip of filter paper; then, before the culture dries up, the preparation is inverted on a drop of a saturated solution of Sudan III in 75 per cent alcohol and examined. Sudan black B in ethylene glycol, usually successful by this technique in demonstrating lipids in many other bacteria,

2 days old at 36 C, and the cells were prepared for observation as for figure 1. Note the density and structure of the slime. The dark areas that appear like dividing nuclei were never observed in slime-free preparations; they probably represent fragments of slime, or they may be a compound picture of slime and intracellular bodies.

Figure 3. Part of a microculture 4 days old at 37 C, grown on a collodion film supported by a solid medium consisting of the medium of Dubos *et al.* + 2 per cent of agar. The preparation was fixed with 95 per cent alcohol for 30 minutes, stained with Meyer's methylene blue, and mounted in water. Note the deeply stained nuclei and lightly stained cytoplasm.

Figure 4. Part of a microculture 1 week old at 37 C. Medium as for figure 3. The preparation was fixed with 95 per cent alcohol for 30 minutes, treated with normal HCl at 60 C for 10 minutes, and stained with Giemsa's solution diluted five times. Note that the nuclei are demonstrable in most of the cells, but that often their form is somewhat altered and the cytoplasm tends to stain more deeply, indicating the liberation and diffusion of some deeply stainable substance, probably nucleic acid, by the action of the acid.

Figure 5. Part of a microculture 4 days old at 37 C, grown as for figure 3. Note the absence of slime. In contrast to figures 1 and 2, cellular organization is here clearly revealed. Note the nuclei, vacuoles, and cell wall. Note also that a nucleus is deformed whenever it is contiguous to a vacuole, indicating that in the living state the vacuole is under a strong turgor pressure.

fails with this organism unless the cells have been subjected to the action of hot water, e.g., when the collodion culture is floated on water at 80 C. This is the first instance, in our experience, when a return to the old technique of demonstrating lipids was made necessary by the impermeability of the cell to Sudan black B in ethylene glycol, a reflection of the chemical or physical characteristics of the cell wall of this organism. Protein was demonstrated by the Sharp test (Knaysi, 1942). The gram procedure used was that of Burke modified by omission of the sodium bicarbonate. Acid fastness was determined by staining with a hot Ziehl-Neelsen's solution and decolorizing with 3 per cent HCl in 95 per cent alcohol.

The test with desoxyribonuclease. Tulasne and Vendrely (reported by Boivin, 1947) showed that the hyperchromatic bodies that persist in the cells of *Escherichia coli* after treatment with ribonuclease are removed by treatment with desoxyribonuclease. In a paper on chicken erythrocytes, which appeared while this phase of the work was in progress, Ely and Ross (1949) stated: "The enzyme attacked cells killed by heat, formaldehyde, alcohol, Carnoy's fixative, and ultraviolet light. Apparently the method of killing the cell makes little difference; it appears to be only necessary that the cell be dead for the enzyme to act." Using *Escherichia coli* in working out the technical details of treating our tubercle bacillus with the enzyme, we soon found that fixation for 30 minutes in 95 per cent alcohol was unsuitable not because it prevents the action of the enzyme but because desoxyribonucleic acid disappears from the treated cells as well as from the controls. Apparently, "depolymerizing" enzymes are present in *E. coli*, and these are not destroyed by alcohol. On the other hand, when the cells are exposed to water at 80 C for 10 minutes, desoxyribonucleic acid disappears only from the cells treated with desoxyribonuclease. Our technique was, therefore, as follows: The collodion cultures were floated on water at 80 C and kept at that temperature for 10 minutes; they were then picked on 22-by-40-mm cover glasses and allowed to dry in the air before being placed in the enzyme or in the control solution. The enzyme solution contained about 5 mg of the crystalline enzyme (purchased from the Worthington Chemical Company, Freehold, New Jersey) to 100 ml of distilled water; since this solution has a pH of about 5.4, a small amount of sterile, neutral broth was added to raise the pH to about 6.8. The control solution was distilled water to which sterile, neutral broth was added in the same proportion. The pH of the two solutions usually differed by about 0.2. The preparations were incubated in these solutions for 30 minutes or 1 hour at 45 C. It should be added that preparations thus treated leave much to be desired in cleanliness and clarity and convinced us of the desirability of further development in the technique of using enzymes for the study of internal structure.

Instruments. Observations with the electron microscope were made with the EMU model of the RCA microscope, often using the double lens recently described by Hillier (1949). The light microscope observations were made with a Zeiss microscope using a combination of 90× apochromatic objective (N.A. = 1.40) and a 20× compensating ocular, and recorded on a 24-by-36-mm, pan-

atomic-x film in a contax camera having a special attachment to the microscope. This convenient method of recording observations is sometimes limited by the resolving power of the film, since the primary magnification in the negative is only 500. In the present work, we had difficulty at first in recording the structure of the intracytoplasmic granules, but this difficulty was finally overcome by selecting a cultural stage in which some of the granules are relatively large.

OBSERVATIONS WITH THE ELECTRON MICROSCOPE

It was pointed out in the previous section that when the Sheard strain of avian *Mycobacterium tuberculosis* is grown in the liquid medium of Dubos *et al.* (1946) and when the cells are centrifuged out, washed with distilled water, and prepared for examination in the old way, the cells, single or in groups, appear as transparent vacuoles inside an electron-scattering mass of slime (figure 1). This slime seems to consist of beaded filaments of variable length apparently formed by polymerization, side to side, of short, rodlike macromolecules denser and slightly thicker at their median part. Under the best conditions, dense, intracytoplasmic bodies may be seen with a variable degree of clarity. Such pictures, revealing as they are regarding the existence of this unusual slime, are of little value for the study of cell structure. Indeed, particles of slime may sometimes adhere to a cell in such a way that they suggest cellular structures and cytological phenomena not really existent (figure 2).

When the organism is grown and prepared for examination by the technique of Hillier, Knaysi, and Baker (1948), the slime may be absent or present to a negligible extent, and the structure of the cell is revealed with a high degree of clarity (figure 5). It is found that the cell is surrounded by a *cell wall*, 230 A thick and ductile to an unusual degree (figure 6). Inside this cell wall, the cytoplasm is surrounded by a very thin *cytoplasmic membrane*, which may be perceived in various figures but the genuine existence of which was demonstrated by the electronic disintegration experiments that will be described below. The system consisting of the cytoplasm and cytoplasmic membrane is highly transparent but not homogeneous; it contains numerous rodlike micelles 50 × 300 A, which resemble those observed by Knaysi and Baker (1947) in *Bacillus mycoides* and may be analogous to those observed by Hillier, Mudd, and Smith (1949) in *Escherichia coli*. The distribution and possible significance of these cytoplasmic components will be discussed below. The cytoplasm encloses two types of bodies which we temporarily label A and C. Bodies of type A are of high density; they are spherical or ellipsoidal and may vary considerably in number, usually 1 to 5 per cell, and in size, 790 to 7,330 by 830 to 8,630 A (figures 5 to 11); particularly in young cultures, they show evidence of division (figures 7 and 10). Bodies of type C are more transparent than the cytoplasm from which they are separated by a thin, dense membrane; they also may vary considerably in size, 500 to 3,000 A, and in number, from a few to 20 per cell (figures 5 to 10); sometimes they appear paired (figure 10). In young cultures, these bodies may be homogeneously transparent or may show evidence of the deposition of a small amount of dense material which was probably in solution.

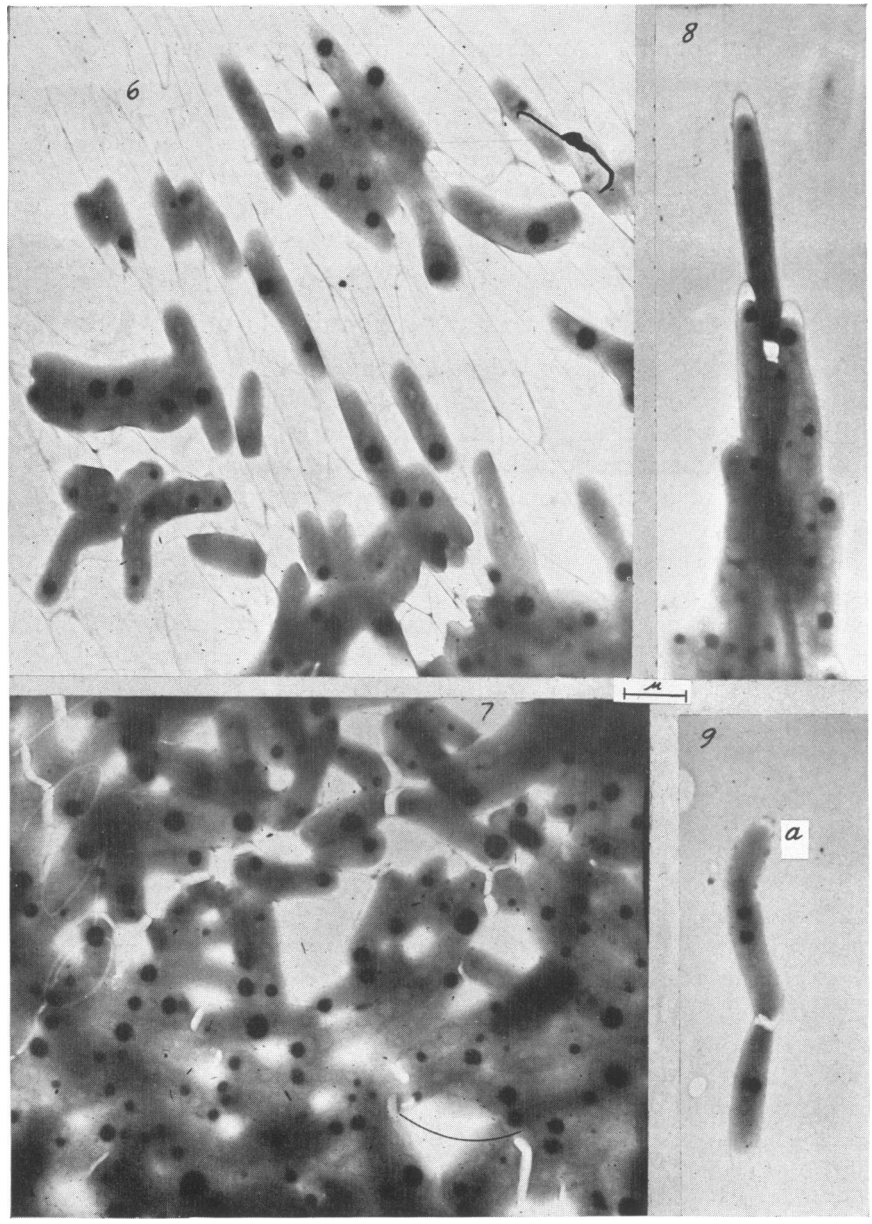

Figure 6. Part of a microculture 6 days old at 37 C, grown as for figure 3. The cells shown grew from two clumps on opposite sides. Shrinkage of these clumps upon drying exerted strong pulling forces that stretched the cell walls without forcing their separation. Note the enormous ductility and stickiness of the cell wall.

Figure 7. Part of a microculture 6 days old at 37 C, grown as for figure 3. Note the wide variation in the size of the nucleus and several large nuclei which, judged by their shape, appear to be dividing. Note also that the cells become shorter and tend to become uninuclear. Note, further, that the vacuoles tend to become smaller and do not appear to contain the spherical micelles.

Figure 8. Part of a microculture 4 days old at 37 C, grown as for figure 3. The micrograph was made with a single lens. Note the apparently dividing nucleus in the cell at the top, and the absence of the spherical micelles from the vacuoles.

Figure 9. Cells from a microculture 6 days old at 37 C, grown as for figure 3. Note that one of the cells, *a*, seems to be without a nucleus.

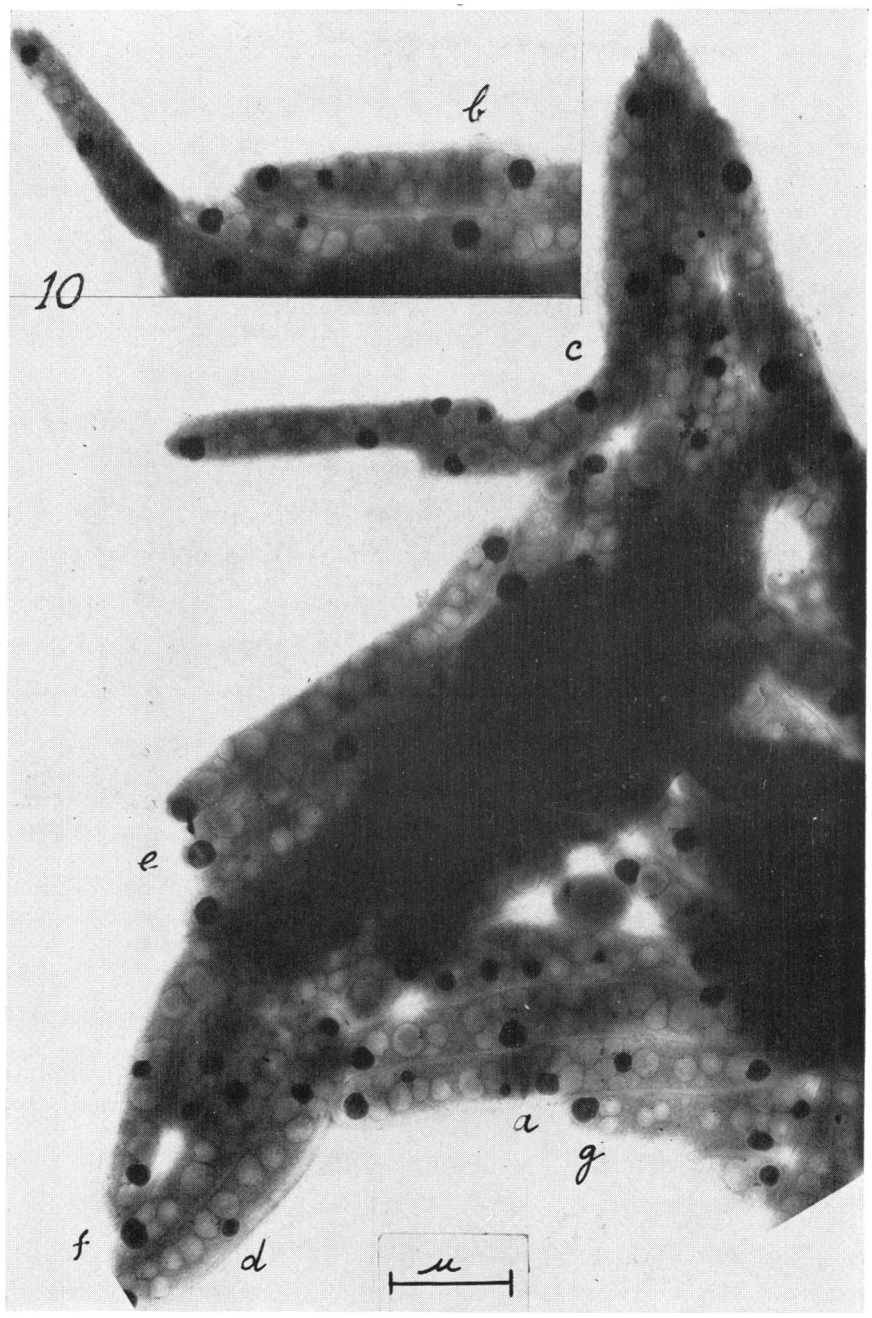

Figure 10. Part of a microculture 21.5 hours old at 37 C, grown as for figure 3. This micrograph shows very clearly the structural organization of the cell into nuclei, cytoplasm, vacuoles, cytoplasmic membrane, and cell wall; the cytoplasmic membrane, however, is clearly visible only in certain cells, for instance cells *a*, *b*, and *d*. The micrograph shows also several cells in various stages of division, and the process appears to be similar to that described in other bacteria (cells *a*, *b*, *c*, and others). It also shows three nuclei, *e*, *f*, and *g*, apparently in the state of division. Note the numerous, rodlike micelles present in the cytoplasm, and the nearly spherical, dense micelles present in some vacuoles, but never in the cytoplasm proper; many of the vacuoles contain amorphous material of unknown nature. Note also the deformation of the nucleus whenever it is contiguous to one or more vacuoles; the outline of the nucleus conforms to that of the vacuole, indicating that the latter must have been, in the living state, under a strong turgor pressure.

As the culture ages, these transparent bodies show more and more of a substance in the form of nearly spherical micelles, 250 to 300 A in diameter (figures 10 and 11). When bodies of both types are contiguous, one usually observes deformation of the bodies of type A to conform with the outline of those of type C; this indicates that, before drying of the cells, bodies of type C were in a high state of turgor (figures 8 and 10). In a number of instances, it appears that there is a greater accumulation of the rodlike micelles around bodies of this type than elsewhere in the cytoplasm, and there was indication that the

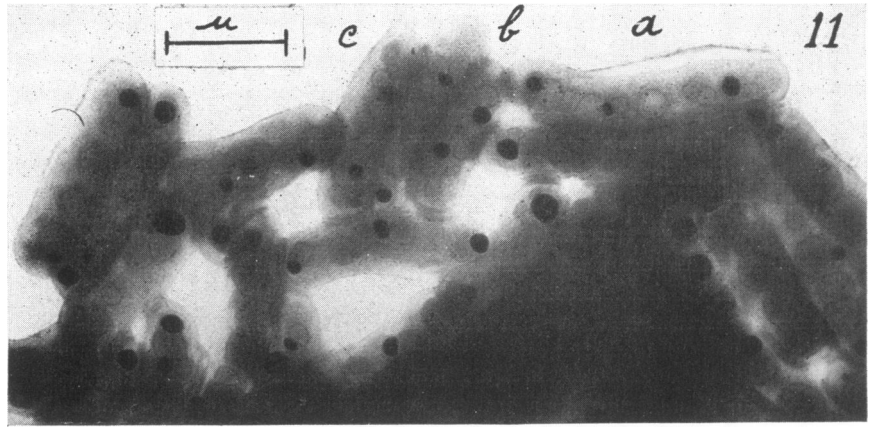

Figure 11. Part of a microculture 2 days + 21 hours old at 37 C, grown as for figure 3. This micrograph was taken with the double lens referred to in the text, and the contrast is generally excellent. The rodlike micelles of the cytoplasm are clearly visible in the original micrograph, and they seem to accumulate around the vacuoles and are indistinguishable in thickness and density from the micelles which form the tonoplast (cells *a*, *b*, *c*, and others); it is suggested that these micelles are surface-active, and that they accumulate at the interfacial surface between the vacuole and the cytoplasm, thus forming the tonoplast or vacuolar membrane. The cytoplasmic membrane is also visible in part of cells *a* to *c* and others and appears also to consist of similar micelles. Note also the dense, spherical micelles present in certain vacuoles. The contrast in this micrograph is such that it is possible to see in marginal cells that slime is not entirely absent but is present in traces.

thin membranes surrounding these bodies are formed by fusion of the rodlike micelles.

Experiments using electronic disintegration. Subjecting a selected area of a preparation in the electron microscope to excessive electron bombardment for short periods was found to cause the bacterial cells to disintegrate, often without rupturing the supporting collodion membrane. The degree of destruction was found to depend on the total bombardment, the time of exposure, and, hence, the intensity; it was also found to depend on the amount of bombardment at moderate intensities given prior to the excessive bombardment. By adjusting these parameters, it was found possible to exercise some control over the disintegration produced and to obtain useful information regarding the architecture of the various structures.

In the strain of *Mycobacterium tuberculosis* presently investigated, it was found that the first structure to disintegrate was the ground substance of the cytoplasm. This was followed by disintegration of the content of bodies of type C, their membrane, and the cytoplasmic membrane. Bodies of type A were very resistant to this treatment. Figure 12 shows a field in which the cells were bombarded with electrons of high intensity. Since electron intensity was not uniformly distributed over the entire field, cells in different locations show different degrees of disintegration. It is highly significant that the process reveals clearly the existence of a cytoplasmic membrane distinct from the cytoplasm in that it resists electronic disintegration, just as it resists autolysis (Knaysi, 1946*a*), much more than the cytoplasm. It is also significant that the rodlike micelles of the cytoplasm persist after the ground substance has disappeared. The process further demonstrates the existence of a membrane around bodies C, and throws considerable light on the structure of bodies A. In cells which have undergone considerable disintegration, or when freed by complete disintegration of the cells, bodies of type A appear to have shrunk in width assuming an oval shape and revealing that these bodies consist of two layers; in two instances the core has disappeared leaving an outer, dense shell.

Cell division. Cells in the process of division were observed, particularly in young cultures (figure 10). The formation of cell plate, usually in a dense median zone free of bodies of either type, followed by centripetal separation of the sister cells may be clearly observed. The process is similar to that described in other bacteria including a human strain of *Mycobacterium tuberculosis* (Knaysi, 1941, 1949).

OBSERVATIONS WITH THE LIGHT MICROSCOPE

The chief purpose of this phase of the investigation was to study the microchemistry of the bodies of type A and to gain further knowledge about their behavior. A wider objective was to learn as much as possible about the cytology of the organism. Since the existence and nature of bodies C were clearly demonstrated with the electron microscope, no attempt was made to make a further study of these structures.

Staining properties. The only readily and deeply staining constituents of the cell with basic or neutral dyes were bodies A and the cytoplasmic membrane. The slime in which the cells are often embedded also stains readily and with surprising depth. The cytoplasm usually stains so poorly that in the acid-fast stain, counterstained with Loeffler's methylene blue, cells which are not acid-fast are very difficult to see. The cytoplasm stains with sufficient depth only when a mordant is used. Bodies A stain deep violet with Giemsa's solution, dark blue with Wright's stain and freshly prepared solutions of Meyer's methylene blue, and dark red with Meyer's basic fuchsin. The cytoplasm usually assumes a purplish tinge with Giemsa's solution. The slime stains purple with Giemsa's solution and red with Loeffler's methylene blue. In preparations fixed for 30 minutes with alcohol, mordanted for 3 hours in 2.5 per cent ferric alum,

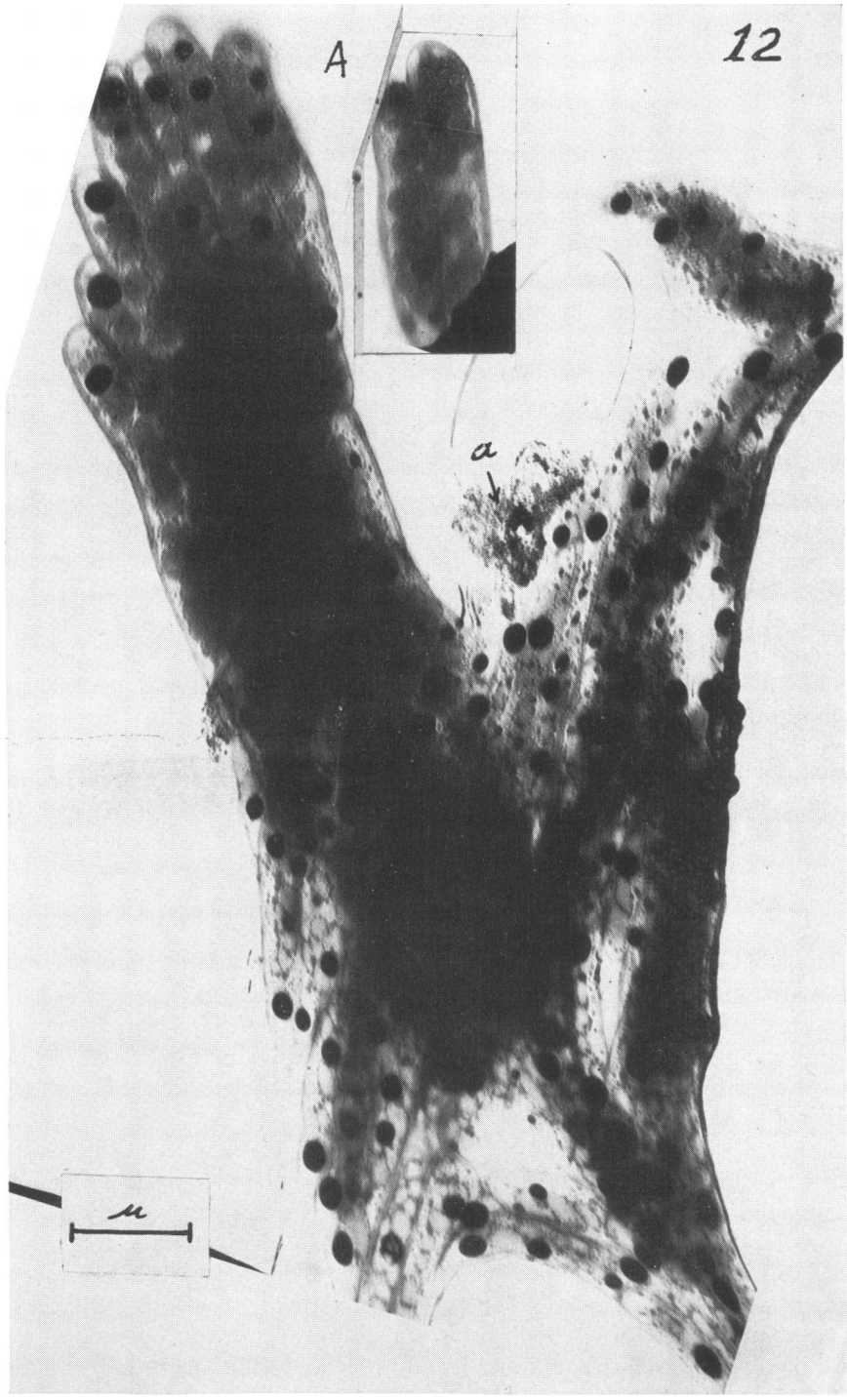

Figure 12. Part of a microculture 21.5 hours old at 37 C, grown as for figure 3. The cells were suddenly exposed to an electron beam of high intensity and were disrupted to a variable extent. Note that the ground substance of the cytoplasm is the first to disappear, and this allows a clearer vision of the rodlike micelles, cytoplasmic membrane, and tonoplast. The relationship of these micelles to both structures is also more clearly suggested than in intact cells (group A). The nuclei appear to be highly resistant to this treatment, but they become more elongated, probably because of partial disintegration of the central core. One nucleus, *a*, is split, and only the resistant cortex remains.

and stained overnight with Delafield's hematoxylin, diluted 1 to 100, most of the cells remain unstained, a few cell groups appear dark blue and beaded, and the slime stains blue. The stained cells appear not to be surrounded by slime (figure 13). In the stained cells, bodies A retain their color when the preparation is mounted in a drop of 2.5 per cent ferric alum.

Microchemical reactions. Microchemical studies were largely restricted to bodies of type A. These bodies give a definitely positive, although weak, reaction by the Sharp test for protein (figure 14). They give a strong lipid test with Sudan III (figure 15), and the lipid material appears to be located in a cortical layer of a body. The Feulgen reaction, like staining with hematoxylin, is strong in a small proportion of the cells (figure 16), and weak or negative in the great majority. In all these tests the results seem to be conditioned by the degree of permeability of the cell to the reagent (see "Media and Methods").

The technique of treating the cells with desoxyribonuclease produces considerable destruction even in the control preparations, and many of the bodies may be found strewn, free, throughout the background. Nevertheless, careful study showed that, in preparations treated with desoxyribonuclease, bodies A, free or still in their respective cells, lose the ability to stain deeply. With Giemsa's solution, for instance, the bodies take a purplish tinge instead of a deep violet color (figure 17). In the control, the violet color generally remains (figure 18). It is interesting and significant that, in preparations treated with desoxyribonuclease, bodies A still give a positive lipid test with Sudan III (figure 19).

Behavior and structure of the bodies of type A. Our observations with the light microscope regarding the number, form, size, and location of bodies A are in good agreement with those we made with the electron microscope. We also found many cases of elongation and pairing usually interpreted as evidence of division (figures 22 and 23). In young cultures and in a high proportion of the cells of mature and old cultures, these bodies are about 0.3 by 0.4 μ and appear homogeneous. However, many cells in old cultures contain bodies, often one per cell, which may be considerably larger; for instance, the bodies shown in figure 20 have the dimensions of 0.8 by 0.8 μ and 0.6 by 1.0 μ. This was also observed with the electron microscope. There is no indication that the large bodies are formed by the fusion of smaller ones, and we are inclined to believe that they develop by the growth of single bodies. These large bodies are structurally differentiated into a cortex that stains dark violet and a core that stains red with Giemsa's solution (figure 20). This structural differentiation was further confirmed on living cells with the phase difference microscope.

Alteration in acid fastness. When the strain used in this work is grown in the liquid medium of Dubos *et al.* (1946), the proportion of acid-fast cells gradually increases until, in a few days, over 90 per cent are acid-fast. On the other hand, when the organism is grown in the same medium to which a small amount of benzimidazole was added, the proportion of acid-fast cells gradually decreases until, after a few days, the entire population is non-acid-fast. Data on this phenomenon are given in table 1. This is also generally true for collodion cultures

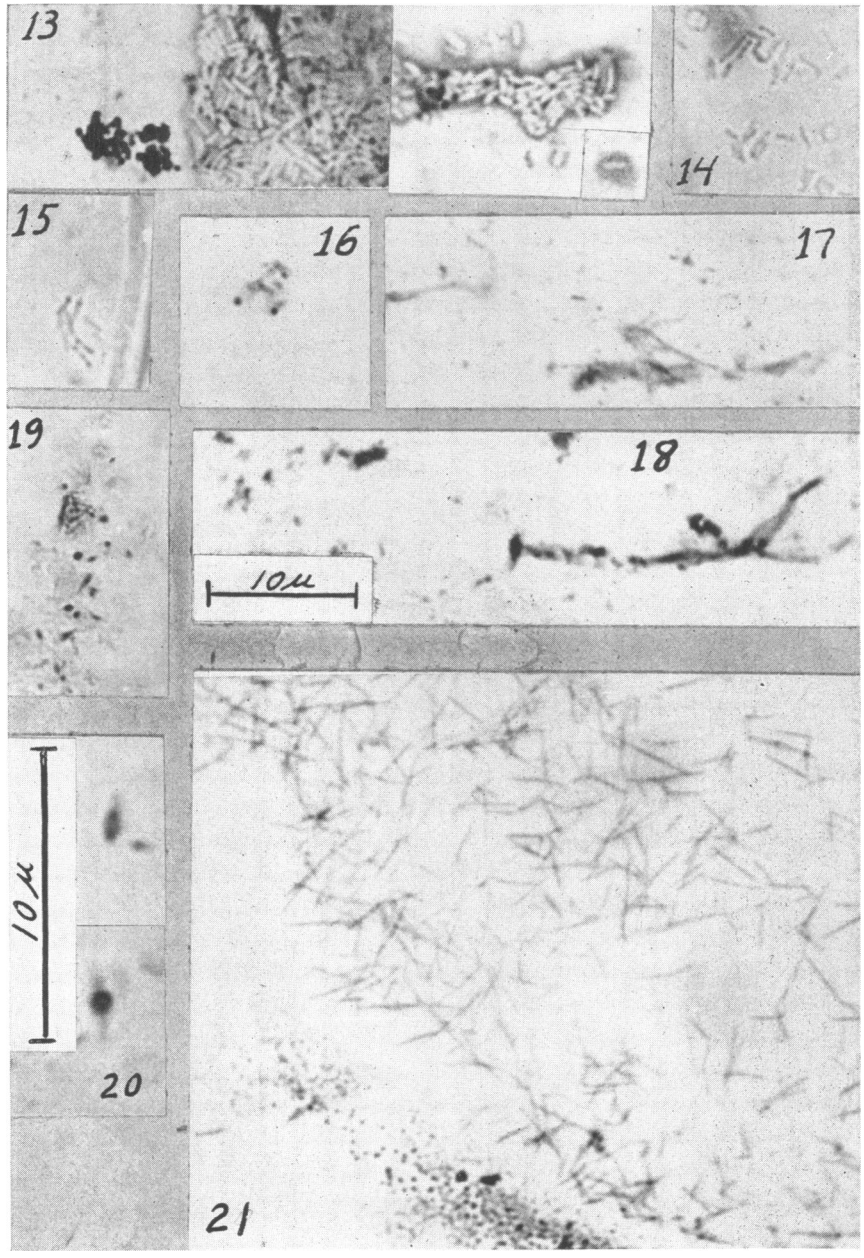

Figure 13. Part of a microculture 12 days old at 37 C, grown as for figure 3. The preparation was fixed with 95 per cent alcohol for 30 minutes, mordanted for 5 hours in 2.5 per cent ferric alum, stained overnight with Delafield's hematoxylin (1 per cent), and photographed mounted in water. Note the deeply staining slime and unstained cells. The few cell groups that are stained appear not to be surrounded by slime. It is also possible that the stained cells were partly autolyzed at the time the preparation was made. The general unstain-

on the solid forms of the two media. Indeed, in a 3-day-old collodion culture on a solid medium, made by adding 20 g of agar and 1 g of benzimidazole to a liter of the medium of Dubos *et al.*, the population was not only entirely non-acid-fast but the great majority of the cells were *gram-negative* (figure 21). There were also gram-negative cells containing gram-positive bodies of type A (figure 21).

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ability of the cells, however, is undoubtedly due to inability of the dye to enter the protoplasm. The scale is the same as for figure 18.

Figure 14. Part of a microculture 1 week old at 37 C, grown as for figure 3. The preparation was fixed with 95 per cent alcohol for 30 minutes and subjected to the Sharp test for protein. Note that the nuclei give a positive reaction. The scale is the same as for figure 18.

Figure 15. Part of a microculture 1 week old at 37 C, grown as for figure 3, and stained with Sudan III. Note that the nuclei are strongly positive. The scale is the same as for figure 18.

Figure 16. A group of cells found in a microculture 1 week old at 37 C, grown as for figure 3. The preparation was fixed with 95 per cent alcohol for 30 minutes and subjected to the Feulgen test. Note the strongly positive nuclei in this group. In the rest of the microculture, however, the majority of the cells were entirely colorless. It appears here, as in the staining with hematoxylin, that the generally negative reaction is due to inability of the reduced fuchsin to enter the protoplasm of most of the cells. The scale is the same as for figure 18.

Figure 17. Part of a microculture 5 days old at 37 C, grown as for figure 3. The microculture was floated on distilled water at 80 C and allowed to remain at this temperature for 10 minutes in order to fix the cells and destroy the autolytic enzymes. It was then picked with a cover glass, allowed to dry so that the collodion film stuck to the glass, and incubated for 30 minutes at 45 C in a solution of desoxyribonuclease containing 5 μg per 100 ml. At the end of this period, the preparation was removed from the solution, rinsed with water immersed in 95 per cent alcohol for a few seconds to remove the film of lipid liberated from the cells, and stained for 10 minutes with Giemsa's solution diluted 5 times. Note the poorly stained nuclei in the cells and in the background. The numerous nuclei in the background were liberated from cells broken up by the sudden exposure to hot water. The scale is the same as for figure 18.

Figure 18. Part of a microculture of the same age as that of figure 17 and grown in the same manner. This microculture was treated like that of figure 17 except that it was incubated in water buffered with a small amount of broth instead of the desoxyribonuclease solution. Note that the nuclei, although somewhat disorganized, are stained much more deeply than in figure 17.

Figure 19. Part of a microculture 6 days old at 37 C, grown as for figure 3. The preparation was floated at 80 C and treated with desoxyribonuclease as for figure 17, then stained with Sudan III. Note that the nuclei still give a positive test for lipid. Note also, in one of the large nuclei, that the lipid is present in the cortex. The scale is the same as for figure 18.

Figure 20. Cells from a microculture 2 weeks old at 37 C, grown as for figure 3. The preparation was fixed with 95 per cent alcohol for 30 minutes and stained with Giemsa's solution diluted 5 times. It was photographed mounted in oil. Note the structural differentiation of the two large nuclei into a chromatin-containing cortex and a nucleoplasmic medulla.

Figure 21. Part of a microcolony 3 days old at 37 C, grown as for figure 3 except that the medium contained 1 mg of benzimidazole per ml. Stained by a gram procedure that differs from Burke's method in that the bicarbonate was omitted. Note that most of the cells and nuclei are gram-negative. Note also a few gram-negative cells containing positive nuclei. The scale is the same as for figure 18.

DISCUSSION AND CONCLUSIONS

Bodies A. It has been shown that the bodies of type A are structurally differentiated into a cortex and a core. This was shown by the technique of electronic disintegration in bodies of average dimensions, and by direct observation with the ordinary and phase-difference light microscope in enlarged bodies commonly found in old cultures. The fact that the bodies of average or small dimensions appear homogeneous with the light microscope is undoubtedly due to optical reasons. For instance, the diameter of the spherical body of figure 20 is 0.8 μ and that of its core 0.3 μ; the ratio of the diameter of the core to that of the body is 0.375 or about 0.4. The width of the elongated body is 0.59 μ and that of its core about 0.25 μ, giving a ratio of 0.423 or about 0.4. Assuming that this ratio also holds in the smaller bodies, a body with a diameter or width of 0.3 μ would have a core of about 0.12 μ. The theoretical limit of resolution of our

TABLE 1

Effect of benzimidazole on acid fastness

AGE OF CULTURE	PERCENTAGE OF ACID-FAST CELLS IN THE PRESENCE OF THE INDICATED CONCENTRATIONS OF BENZIMIDAZOLE				
	0.0 mg/ml*	0.01 mg/ml†	0.1 mg/ml*	1 mg/ml*	2 mg/ml*
days					
2	72.9	47.7	49.9	47.1	51.5
4	62.5	76.7	26.9	10.0	0.4
6	72.4	69.0	52.2	14.4	10.0
8	89.7	86.5	72.4	34.9	30.4
10	97.2	78.3	57.8	24.0	21.8
12	86.8	67.3	78.8	39.8	33.6

* Average of three experiments. † Average of two experiments.

objective with central, green light is about 0.18 μ. We believe, however, that the true ratio between the diameter of the core and that of the body is greater than indicated by these measurements, and that interfacial optical phenomena (Richards, 1948) and the fact that the core is surrounded by a dense, deeply stained cortex contribute to making the core appear smaller than it really is. Indeed, the cortex appears to be much thinner in electron micrographs than in photomicrographs, but no significant comparison can be made because the electron micrographs represent a structure simply dried and the photomicrographs a structure stained and mounted in water. A similar discrepancy has been consistently found between electron and light microscope measurements in the case of the cytoplasmic membrane (Knaysi, 1949).

The cytochemical and staining reactions of bodies A show that the cortex consists of chromatin in which the desoxyribonucleic acid is associated with both protein and lipid. The nature of the core is unknown; it stains red with Giemsa's solution and is less resistant than the cortex to electronic disintegration. The bodies sometimes appear elongated or paired (figures 22 and 23), which is usually interpreted as evidence of division, especially in growing cultures. How-

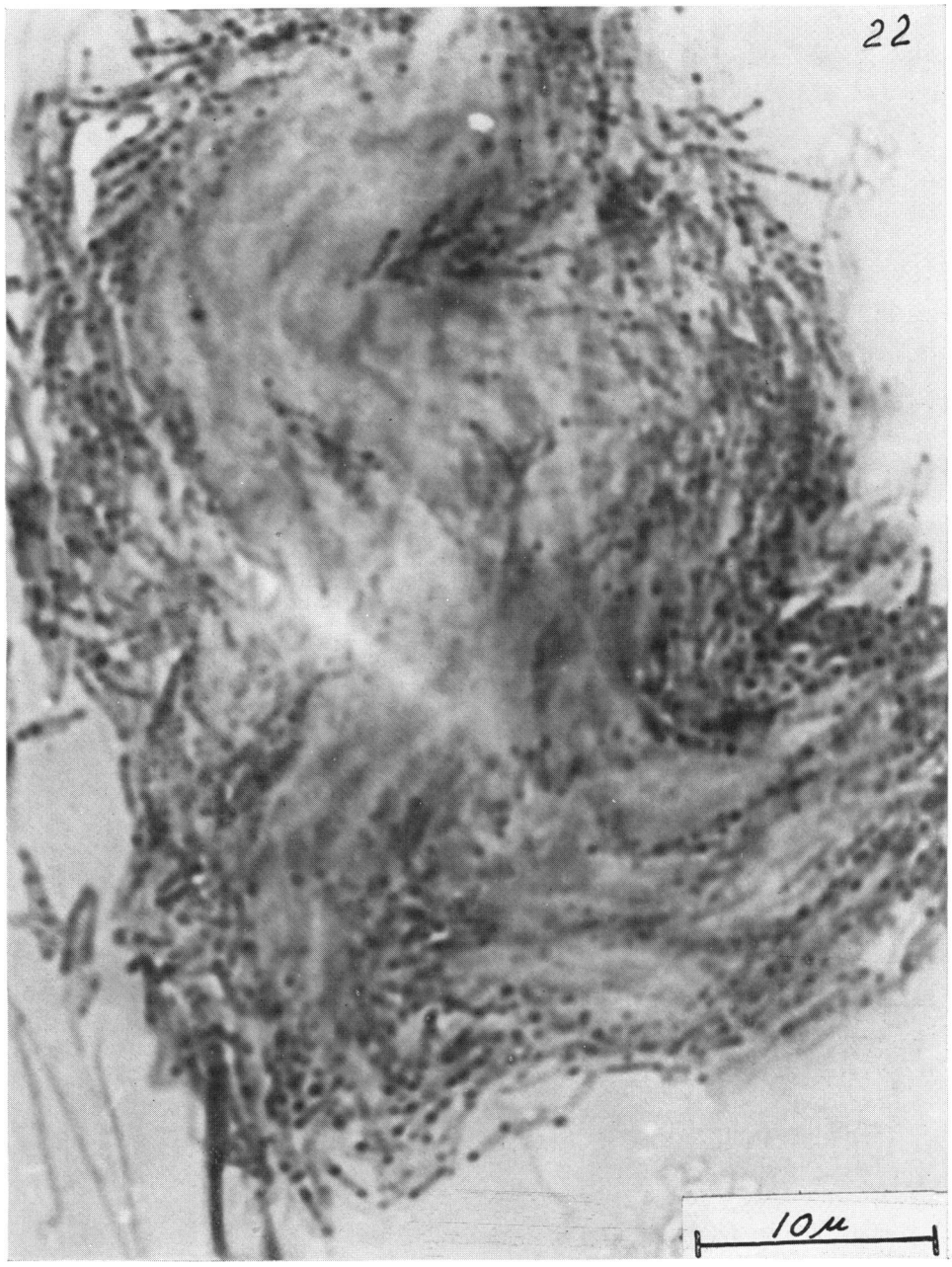

Figure 22. A microcolony 3 days + 18 hours old at 37 C, grown as for figure 3. The preparation was fixed with 95 per cent alcohol for 30 minutes and stained with Giemsa's solution diluted 5 times, for 10 minutes. This picture shows many elongated or paired nuclei.

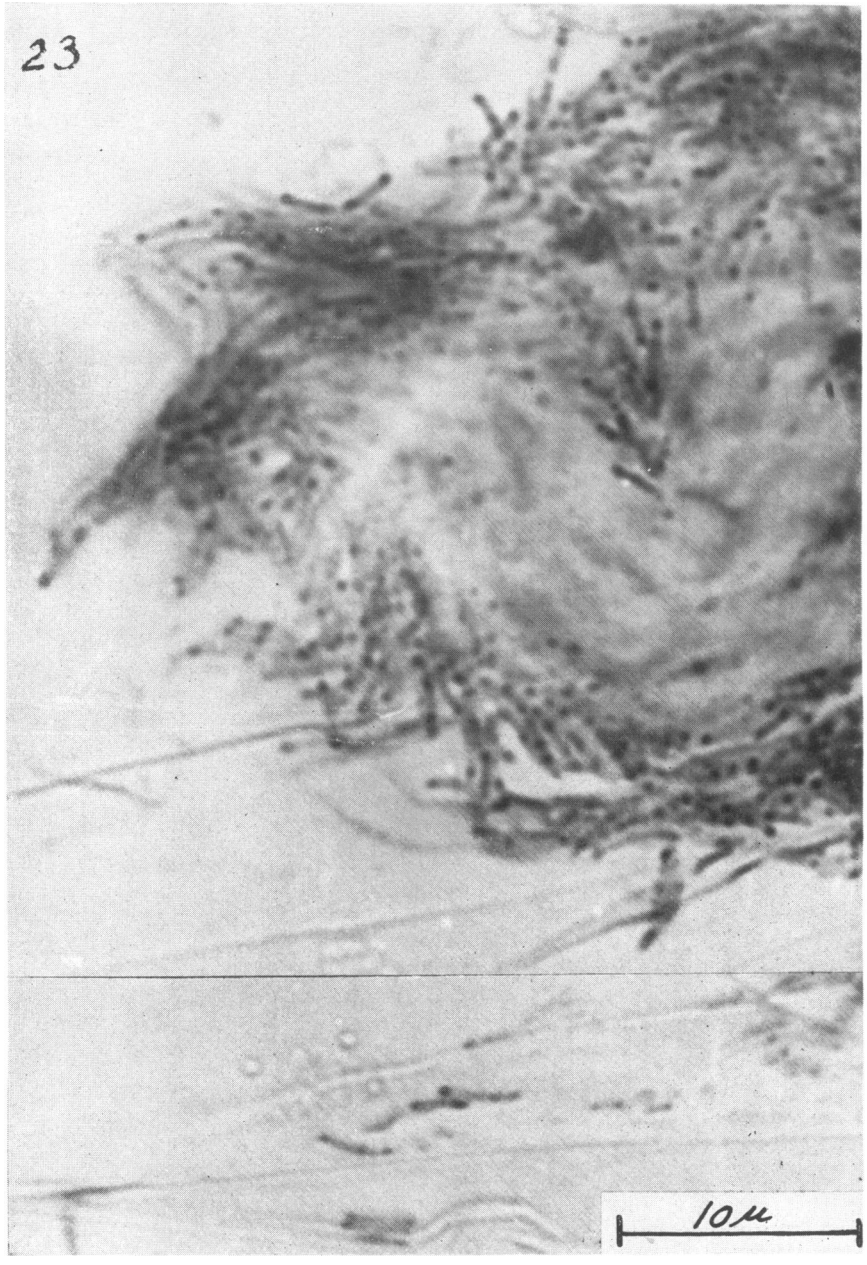

Figure 23. Parts of microcolonies of the same age as that of figure 22 and similarly grown and stained. Note the presence of elongated or paired nuclei.

ever, because of the slow rate of growth of the organism, the proportion of elongated or paired bodies as well as that of dividing cells at any given moment remains low. In old cultures, the evidence of division is of much lower frequency,

and the bodies appear to grow in size so that a single body often occupies an entire cell or the major part of it, a phenomenon also observed in *Escherichia coli* in old cultures or in cultures in which aging was experimentally induced. In this sense it may be said that the entire cell is a nucleus. On the basis of the chemical composition of the bodies of type A and of what we consider as evidence of their multiplication by division and their constant presence in practically all cells regardless of cultural age (rarely, cells like *a* of figure 9 were found which did not contain bodies of this type, but there is no assurance that such cells are viable), and in spite of the absence of a recognizable life cycle in which behavior could be further followed, we are led to the conclusion that *these bodies are of nuclear nature*. Indeed, there are no other bodies in the cell which could be called nuclei. It will be shown below that the bodies of type C do not possess nuclear characteristics; nor could the rodlike micelles present in the cytoplasmic system be chromidia since, unless the nuclear material of *Mycobacterium tuberculosis* is different from that of all other organisms including certain bacteria in which nuclei have been demonstrated, they would have conferred on the cytoplasm staining and microchemical reactions that it does not possess. Since the structure of this organism is clearly demonstrable with both the electron and light microscopes without previous treatment, chemical, physical, or physiological, this argument and the observations on which our conclusion is based acquire a special force.

The existence of lipid apparently as a part of chromatin needs further comment. Knaysi (1929), whose observations on an avirulent strain of the human variety are, as far as they went, in substantial agreement with the present ones, had demonstrated lipid in these bodies, and that was the chief reason why he did not attribute to them a nuclear nature. He, however, had observed their constant presence and evidence of their division and never considered them as inclusions of reserve material as did other investigators. In view of the peculiarity of *Mycobacterium* with respect to lipid content, it should not be considered unlikely that their chromatin also would contain lipid; perhaps it will be found that some lipid is also present in the chromatin of certain other bacteria.

A claim has been made recently that, besides nuclei, the bacterial cell contains polar bodies which may play a role in the life of the cell. We found no evidence for the existence of polar bodies distinct from the nuclei in the organism investigated. Bodies A which, we just showed, have a nuclear nature may occupy a polar position, be located near the middle, or be distributed throughout the cytoplasm. We found this also to be true with *Escherichia coli*, in which it is not uncommon for a nuclear body to occupy a polar position. The same was found true for *Bacillus mycoides* (Knaysi and Baker, 1947). Consequently, until experimental details are published, and unless they are satisfactorily confirmed, we shall continue to consider polar bodies of nonnuclear nature as something that may be formed by cells under certain conditions, often related to unusual cell plate formation (see Knaysi, 1946*b*) or that has the nature of vacuoles or inclusions.

Hillier, Mudd, and Smith (1949) recently observed, in actively growing cells

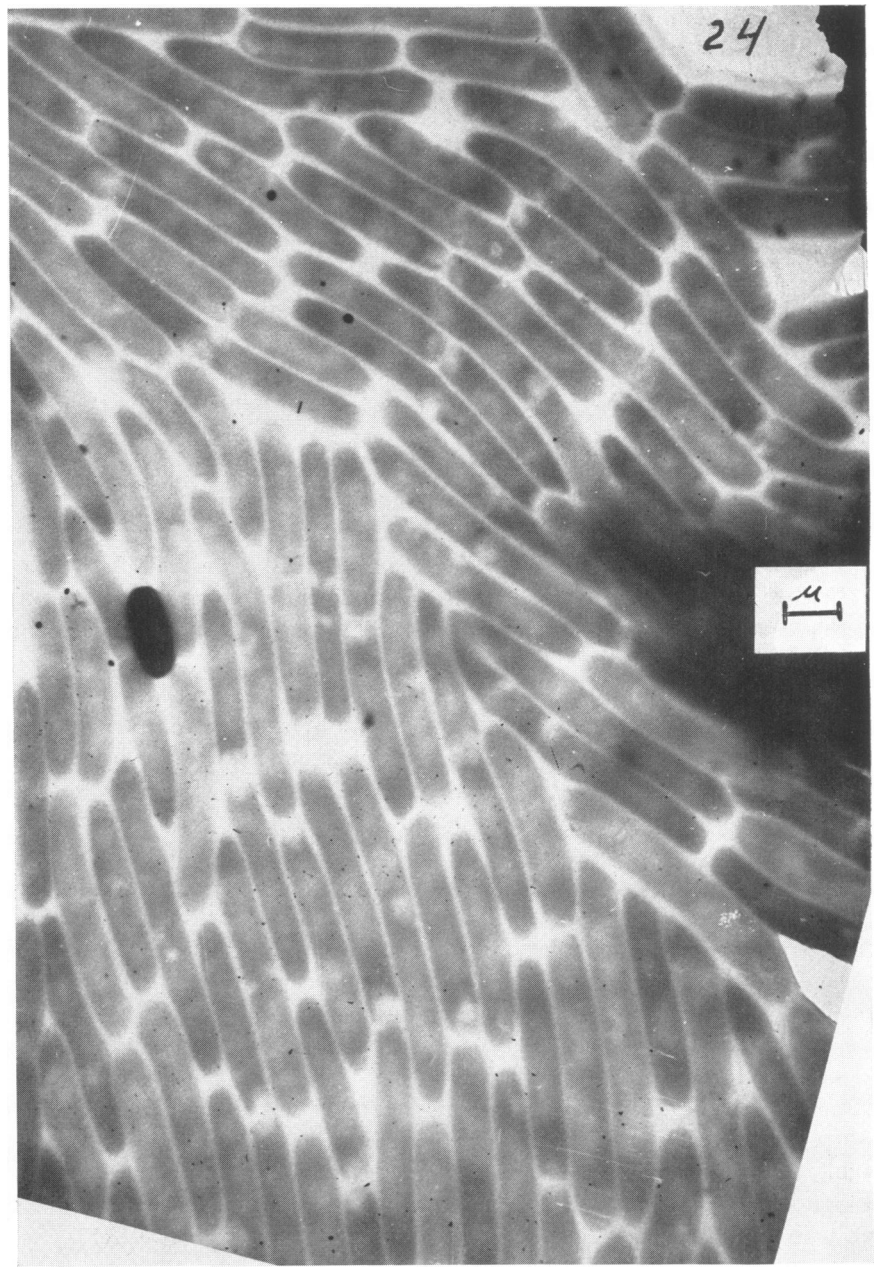

Figure 24. "*Bacillus agri*," strain 13. Part of a young microculture grown as for figure 3. Note the transparent rings, dots, or spots of various sizes, forms, and locations. These are, in some way, related to the nuclei. Rings and large spots probably represent a group of overlapping dots.

of *Escherichia coli*, bodies that are transparent to the electron beam and that by their form and arrangement resemble the nuclear bodies demonstrable in similar cells by staining with neutral or basic dyes after treatment of the cells by acid as in the Feulgen technique. Bodies of this type were not demonstrable in the strain of *Mycobacterium tuberculosis* investigated. On the other hand, transparent bodies which, we believe, are related to rapidly dividing nuclei were clearly seen in actively growing cells of a strain of "*Bacillus agri*" (figure 24) and may be seen within the dividing nuclei of *Bacillus mycoides* (strain C_2). In one instance, these bodies were seen in a row across the median part of the dividing nucleus (figure 25), and the impression given is that of a nucleus in the stage of metaphase. Whether these bodies are chromosomes, genes, or subsidiary bodies formed during nuclear division cannot be said at the present time.

Bodies of type C. Bodies C, which are readily observed with the electron microscope in collodion cultures, are not easily demonstrable with the light microscope in the strain investigated; they do not stain with basic or neutral dyes and could be distinguished only by contrast in a well-stained cytoplasm, a condition rarely realized in the strain investigated. Furthermore, we feel that these bodies are delicate and easily destroyed by manipulations; this may be the chief reason why they were not demonstrable with the electron microscope in specimens prepared by the old technique of centrifuging and washing. Nevertheless, we did occasionally observe bodies of this type, and we believe that we could have worked out a suitable technique for their demonstration. This was considered unnecessary since these bodies were observed by Knaysi (1929) in a human strain, and his illustrations bear considerable resemblance to our electron micrographs.

Since the bodies of type C are unstainable, are highly transparent to the electron beam, exert turgor pressure, and accumulate a solid content that tends to disappear in old cultures, these bodies are to be considered cell sap vacuoles. Whether they are not formed in submerged cultures or whether they are formed and subsequently destroyed by manipulation it is not possible to say definitely at the present time. The fact that they were not observed by previous investigators who used surface cultures and that they are demonstrable in droplet cultures where the organism grows somewhat as in submerged cultures makes the second alternative appear the more likely.

The rodlike micelles. Knaysi and Baker (1947) observed rodlike particles in the cytoplasmic membrane of *Bacillus mycoides*. Hillier, Mudd, and Smith (1949) considered the ones they observed in *Escherichia coli* located in the cytoplasm proper. In the present work, morphologically similar rodlike particles appear to be distributed in the cytoplasmic membrane and in the cytoplasm. These particles may be large, complex macromolecules or they may be the result of the aggregation of somewhat smaller molecules brought about by desiccation. In any case, they appear to exhibit high surface activity, accumulating around the vacuoles and probably forming the tonoplast (vacuolar membrane). Indeed, they appear to be integral components of the cytoplasm and, because of their surface activity, accumulate at interfacial surfaces forming membranes. This

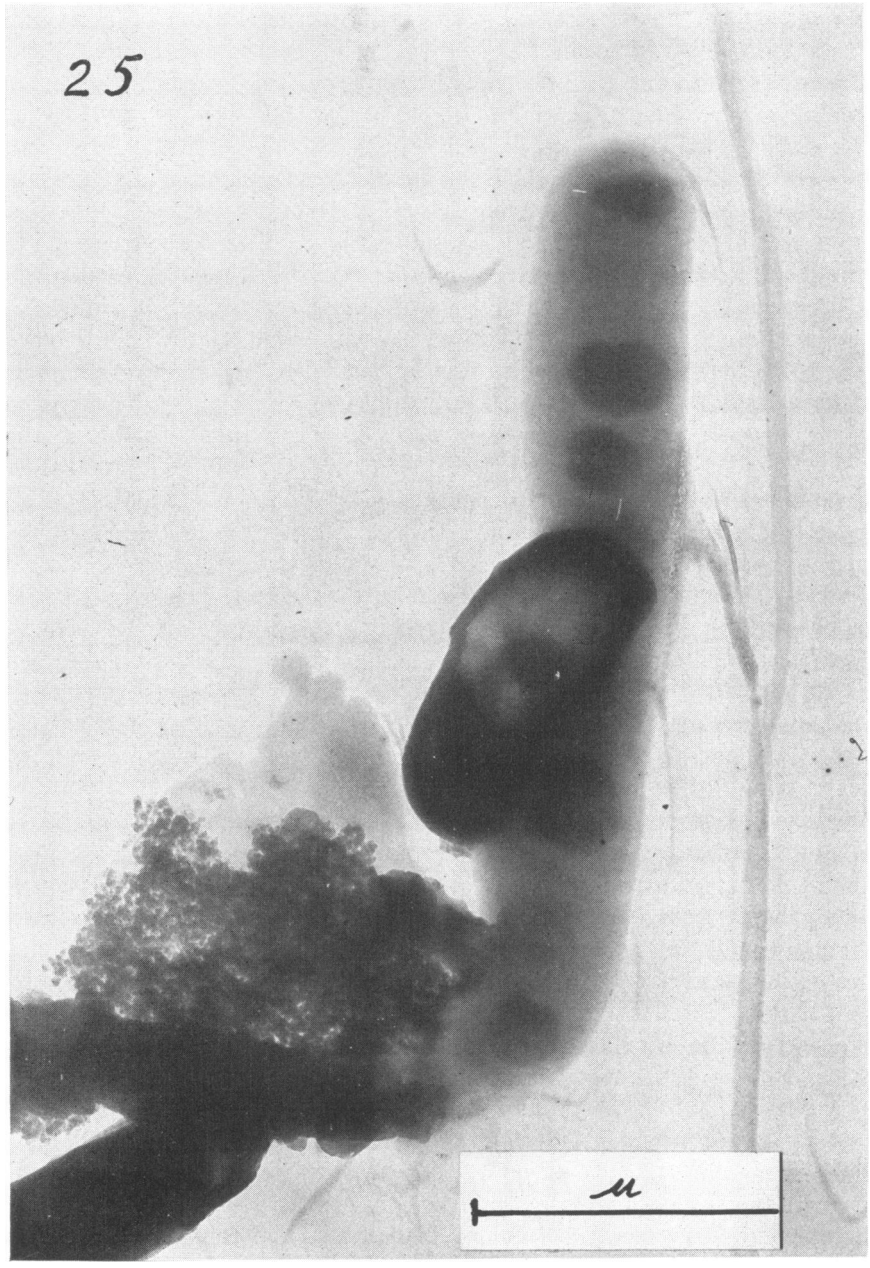

Figure 25. Bacillus mycoides, strain C_2. Part of a microculture 16 hours old at 25 C grown on a collodion film floating on a solution of glucose and sodium acetate. The microculture was dried from the frozen state. Note the presence of transparent dots within the nuclei. In one of the nuclei, the dots appear as a row in the median part of the nucleus.

hypothesis, now suggested by visual observation of the individual micelles, is fundamentally similar to the one postulated by Knaysi (1938, 1944) about the origin of both the tonoplast and the cytoplasmic membrane based on the similarity of their chemical composition. Accordingly, these micelles are expected to contain lipid and protein, possibly in association with other complex radicals.

The action of benzimidazole. A small amount of benzimidazole added to the medium of Dubos *et al.* gradually renders the cells non-acid-fast and gram-negative. Benzimidazole is said to inhibit the synthesis of ribonucleic acid (Woolley, 1944), which furnishes an explanation of the alteration in the gram reaction. On the other hand, acid fastness has always been associated with lipids. Although microscopic tests with Sudan III show that the nuclei and cytoplasm of cells grown in the presence of benzimidazole appear to contain as much lipid material as those of cells grown without benzimidazole, it is possible that benzimidazole inhibits the synthesis of a specific lipid. It is also possible that the mechanism of acid fastness is more complex than is generally believed.

Remarks on extracellular slime. The two fascinating features of the Sheard strain of *Mycobacterium tuberculosis* are, first, that its cytoplasm is practically unstainable with the basic and neutral dyes tried and is highly transparent to the electron beam, within the scope of our work, regardless of the medium and the age of the culture; second, that it is surrounded by slime which is highly stainable and opaque to the electron beam. When we remember the numerous techniques devised to render bacterial slime stainable, or demonstrable with the electron microscope, the latter property appears to be truly remarkable.

The slime does not give the lipid test with either Sudan III or Sudan black B, which confirms the early observations of Knaysi (1929) on a human strain. On the other hand, it stains red with Loeffler's methylene blue, an alkaline solution that was prepared several weeks before its use. This is a recognized property of volutin, which is chiefly ribonucleic acid, a substance that is usually present, mostly combined, in the cytoplasm and is now known to be the cause of the usually deep staining of bacterial cytoplasm. Could it be possible that the situation is somewhat reversed in the case of the strain investigated? That ribonucleic acid, in some combination, is extraprotoplasmic, or that it is formed in the protoplasm and eliminated to the cell surface? These questions deserve further investigation.

SUMMARY

The structure and microchemistry of the Sheard strain of *Mycobacterium tuberculosis* var. *avium* were studied with the electron and light microscopes. The cell consists of a thin, highly ductile cell wall, a thin cytoplasmic membrane, a poorly stainable cytoplasm containing many rodlike micelles of unknown nature, a number of cell sap vacuoles (several to 20), and 1 to 5 nuclei (mostly 2 to 4). Dimensions of these structures are given. The cell sap vacuoles contain material in solution and, usually, nearly spherical micelles, apparently a by-product of metabolism. The resting nucleus consists of a lipid-containing chromatin layer and a core that stains red with Giemsa's solution. A similar structure was ob-

served in the resting nucleus of *Escherichia coli*. In submerged cultures, the cells are embedded in slime of unusual properties; it is highly stainable and highly opaque to the electron beam. Old techniques are evaluated and new techniques of electron and light microscopy are given and discussed in their relation to the physicochemical properties of the cell and cellular structures.

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