EVIDENCE SUGGESTING THAT THE GRANULES OF MYCOBACTERIA ARE MITOCHONDRIA[1]

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The granules which are so readily demonstrable in mycobacteria were interpreted by Robert Koch (1884) as spores.[4] More recently they have been interpreted, with minor variations, as reserve particles, fat droplets, degeneration products, reproductive (Much) granules, conidia, gonidia, "sporide," and nuclei. The literature has been well reviewed among others by Wessel (1942), Knaysi (1929), and Knaysi, Hillier, and Fabricant (1950).

The present work demonstrates that these granules are loci of enzymatic oxidative-reductive activities, contain phospholipid, and give in high dilutions of Janus green B the succession of colors characteristic for the staining of mitochondria. The granules have smooth spheroidal to ellipsoidal contours and, except when deformed by adjacent structures, have surfaces of minimal areas. When the granules have been volatilized by intense electronic bombardment, definite limiting surface membranes remain. The essential attributes of these granules are characteristic of the mitochondria of the cells of higher animals and plants.

METHODS

The organisms used for this study were *Mycobacterium thamnopheos* (Aronson, 1929), *Mycobacterium tuberculosis* var. *hominis*, strain H37Rv, and three cultures from the sputum of patients, proven to be virulent tubercle bacilli by guinea pig inoculation. All cultures were obtained from the Henry Phipps Institute for Tuberculosis. The organisms were cultured on 5 per cent glycerol agar using an extract broth base for *M. thamnopheos* and a fresh infusion broth base for the virulent human tubercle bacilli. *M. thamnopheos* served as the pilot organism upon which the various techniques were worked out prior to their application to the virulent human strains.

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[4] "Die Sporen sind eiförmig, am Rande von einer feinen gefärbten Linie begrenzt und finden sich gewöhnlich in einer Zahl von 2–6 in einem Bazillus."

Preparations made from organisms grown on a collodion film on the surface of an agar plate were cleaner than those grown on agar alone, and there was no observable difference in the organisms. Consequently, following the technique described by Hillier, Knaysi, and Baker (1948), a thin collodion film was placed on the plates. A sterile Pasteur pipette was found convenient to inoculate dropsize areas on the collodion film, thus enabling the use of one cell suspension for several plates. When the excess fluid was removed from the drop and the remainder allowed to dry, the inoculation area was readily discernible as a discrete round area on the collodion.

Light microscopy. For light microscopy a Bausch and Lomb research microscope CCTB with balcoated lenses, a 15× eyepiece, and 97× achromatic oil immersion objective of n.a. 1.25 with built-in diaphragm was used. Critical illumination was provided by a Bausch and Lomb research microscope illuminator (ribbon filament lamp) using immersion oil between the condenser and slide as well as the slide and objective. Light microphotographs were taken with a Bausch and Lomb L camera, and the 15× eyepiece of the microscope was replaced by the 12.5× eyepiece, giving a final magnification on the photographic film of 2,425×.

A specimen for light microscopic observation may be prepared as follows: With a scalpel, kept in alcohol, then flamed, a block of agar bearing the collodion with the inoculation area was cut out. This was placed in the vapour of 2 per cent aqueous solution of OsO_4 to fix the cells. Fixation for one minute was used for *M. thamnopheos*, and for two minutes for the virulent human tubercle bacilli. A clean cover slip was placed over the inoculation area and the whole inverted. The agar block was flipped off with the knife point, leaving the collodion film and the inoculum on the cover slip. This was allowed to air-dry for a few minutes. Then the knife blade was dipped in distilled water and with the drop of water that clings to the point the collodion film was peeled off, leaving the inoculum intact adhering to the cover slip. The cover slip was then blotted dry and mounted in clarite or processed further.

Electron microscopy. Specimens for electron microscopy were prepared as follows: A block of agar bearing the dropsize inoculum area on a collodion film was cut from the plate. The agar block, collodion film, and inoculum area were placed in the vapours of 2 per cent aqueous OsO_4 for fixation. The agar block was immersed in a dish of water; the agar sinks to the bottom leaving the collodion film bearing the inoculum floating on the surface. The square of collodion was picked from the water on a wedge-shaped piece of 200 mesh copper screen and placed in a desiccator to dry. To facilitate transportation and storage these pieces of copper screen bearing the electron microscopic specimens were fixed to a microscopic slide-shaped piece of note card. When the electron microscope disk was punched from the copper screen, the note card was used as a backing that offered sufficient resiliency for the punch to cut the copper disk cleanly.

Electron microscopy was conducted with the electron microscope of the Laboratories for Research and Development, the Franklin Institute, Philadelphia, Pennsylvania. Technical assistance was provided by Mrs. Jean Minkin, research

engineer for these laboratories. The electron microscope was an RCA EMU model with biased electron gun.

Reduction of tetrazolium and neotetrazolium. The sites of enzyme activity that tetrazolium demonstrates were shown by incorporating the tetrazolium in the agar on which the organisms were grown. A 1 per cent aqueous solution of tetrazolium chloride[5] was prepared. This may be sterilized by autoclaving at 15 lb for 15 minutes or by filtration. If kept sterile and stored in the dark, this solution remains usable for 3 to 4 months. Agar containing a final concentration of 0.005 per cent tetrazolium was found to give an adequate coloration in about 6 hours. Higher concentrations of the tetrazolium gave coloration more rapidly but were more toxic to the organisms. The plates were incubated at room temperature for *M. thamnopheos*, and at 37 C for the virulent human tubercle bacilli. Since anaerobic conditions were found markedly to increase the reduction of tetrazolium (Brodie and Gots, 1951), plates of *M. thamnopheos* were incubated in anaerobic jars in which the air pressure was reduced to 260 mm Hg and returned to atmospheric pressure with 90 per cent N_2 and 10 per cent CO_2.

Neotetrazolium[6] is more easily seen and photographed due to its dark bluish-purple color on reduction. Agar plates containing 0.005 per cent neotetrazolium were prepared in the same manner as the tetrazolium-containing plates. At this concentration the reduction of neotetrazolium was detectable much earlier than that of tetrazolium. Cells grown on the neotetrazolium-containing agar could be counterstained with carbol-fuchsin (as used for the Ziehl-Neelsen acid-fast stain), diluted 1:2 with distilled water at room temperature for 2 minutes for *M. thamnopheos*, and 8 to 10 minutes for the virulent human tubercle bacilli.

The reduction of neotetrazolium was observed in living cells by placing a small drop of cells suspended in sterile distilled water on a thin film of 0.005 per cent neotetrazolium-containing agar spread on a microscopic slide. The entire preparation was then sealed under a cover slip with melted paraffin and observed continuously under the microscope. This was done with *M. thamnopheos* and *M. tuberculosis* var. *hominis*, strain B.V.

Oxidation of Nadi reagent. The mycobacteria were also examined for Nadi positive areas of cytochrome oxidase activity by adding the Nadi reagent to cell suspensions. The Nadi reagent (Hawk, Oser, and Summerson, 1947) consists of equal parts of a 1 per cent aqueous solution of dimethyl-p-phenylenediamine and a 1 per cent solution of α-naphthol in 95 per cent ethanol. Five drops of each of the above solutions were added to 1 ml of cell suspension. At given time intervals wet and permanent specimens were prepared. For wet mounts a small drop of the cell suspension was placed on a microscopic slide and sealed under a cover slip with melted paraffin. Permanent preparations were made by placing a drop of the cell suspension on a block of dry hard agar and allowing this to soak up the excess moisture. The cells on the agar block were

[5] 2,3,5,-triphenyltetrazolium chloride; obtained from Arapahoe Chemicals, Incorporated, Boulder, Colorado.

[6] Neotetrazolium chloride; Montclair Research Corporation, 4 Cherry St., Montclair, New Jersey.

then fixed in the vapour of a 2 per cent aqueous solution of OsO_4 for 1 and 2 minutes[7] for *M. thamnopheos* and the virulent human tubercle bacilli, respectively. Then the cell-bearing surface of the agar block was inverted onto a cover slip, the agar block flipped off with the point of a knife, and an impression smear remained on the cover slip. After drying thoroughly this was mounted in clarite.

Staining with Janus green B. Michaelis (1900) described the vital stain Janus green B (diethylsafraninazodimethylanilin) as being specific for mitochondria. Since the reduction of tetrazolium and neotetrazolium and a positive Nadi reaction at certain loci indicated enzyme activities typical of mitochondria in other cells and organisms, Janus green B[8] was incorporated in the agar upon which the mycobacterial cells were grown. The final concentration of Janus green B was 0.05 per cent, and the agar plates containing it were prepared and incubated in the same fashion as those containing tetrazolium and neotetrazolium. Janus green B was also incorporated in agar with tetrazolium, both at final concentrations of 0.005 per cent.

The Janus green B staining granules were also demonstrated by preparing a moderately heavy suspension of cells in distilled water to which various amounts of Janus green B were added. The dye was used here in final concentrations ranging from 0.0005 to 0.01 per cent, the approximate range of specificity as indicated by E. V. Cowdry (1918). The lower concentrations are more specific because higher concentrations stain other structures as well as the entire cell. Wet and permanent mounts were prepared as previously described.

Acid-hematin stain. In order to demonstrate the presence of phospholipids in the organisms, we applied Baker's (1946, 1947) acid-hematin[9] stain. This staining procedure is quite long and for details the reader is referred to Baker's paper. Since, according to Baker, the specificity of the stain depends on strict compliance with the conditions he describes, his procedure was strictly adhered to. Impression smears of the organisms were made on cover slips coated with Mayer's albumin fixative and were then fixed and stained according to procedure. Since positive results are indicated only when the pyridine-extraction test is done also, a duplicate set of slides was prepared in the same manner and this latter test applied.

Staining with Harman's stain. Harman's (1950*a*) stain for mitochondria was applied to the organisms as follows: Impression smears on cover slips coated with

[7] The OsO_4 vapour apparently oxidized the dimethyl-p-phenylenediamine and α-naphthol adsorbed on the surface of the cells, for after fixation they were "counterstained" a delicate bluish-purple which made the cells readily discernible, and the very dark bluish-purple Nadi positive granules stood out nicely. This coloration of the cells was not due to enzyme activity on the surface or throughout the cell as we were able to demonstrate with adequate controls.

[8] Janus green B, dye content 54 per cent, certification #CJ-5, the Coleman and Bell Company, Norwood, Ohio.

[9] The hematoxylin for the acid-hematin solution was: Hematoxylin, National, certified; cert. #NH7; Arthur H. Thomas Company, Philadelphia, Pennsylvania.

Mayer's[10] albumin fixative were prepared as described previously. The cover slips were placed in a 4 per cent solution of fast green FCF[11] in 10 per cent aniline water that had been heated to 63 C and allowed to cool while the cover slips were in it. After 8 minutes the cover slips were removed, rinsed in distilled water, and placed in a saturated aqueous solution of picric acid for 10 minutes. Then they were rinsed and placed in 1 per cent aqueous solution of phosphomolybdic acid for 2 minutes. After another rinse in water they were passed at intervals of 2 minutes each through 70 per cent, 90 per cent, and absolute ethanol, an equal mixture of ethanol and xylene, and finally xylene, after which they were mounted in clarite. Preparations were not counterstained with safranin.

Significance of Tests Used. Reactions of Mycobacteria

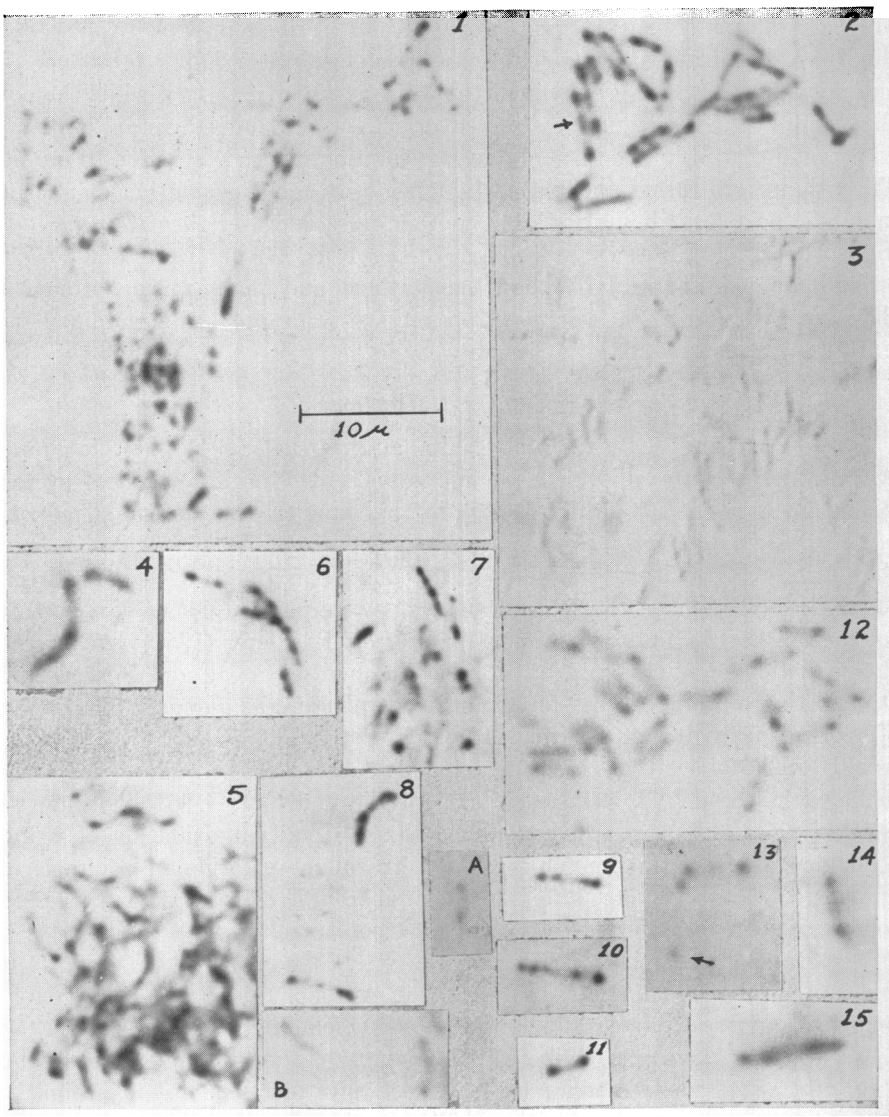
Tetrazolium and neotetrazolium. Kuhn and Jerchel (1941) first introduced triphenyltetrazolium chloride as a reduction indicator in biological systems. Bielig, Kausche, and Haardick (1949), in analyzing the mechanism of reduction of tetrazolium to its red form, formazan, by cells of *Escherichia coli*, showed that maximal activity was obtained at about pH 8.5 and at temperatures up to 70 C; these pH and thermal relationships were pointed out as corresponding to those of the flavoprotein enzyme diaphorase. Pfleiderer (1951) demonstrated the reduction of tetrazolium by isolated diaphorase. Sevag and Forbes (1950) studied the formation of red formazan by *Staphylococcus aureus* in the presence of glucose as hydrogen donator. They observed that even the inactive cells would form formazan if certain nitrogenous substances, i.e., amino acids, were included in the dehydrogenase system. Brodie and Gots (1951) working with isolated yeast diaphorase demonstrated the concomitant oxidation of reduced coenzyme I and reduction of triphenyltetrazolium chloride in equimolecular amounts. Brodie and Gots (1951) also isolated from *E. coli* an oxidase for reduced coenzyme I which reduces tetrazolium and neotetrazolium.

Neotetrazolium has been utilized by Antopol, Glaubach, and Goldman (1948) and by Narahara, Quittner, Goldman, and Antopol (1950) as an indicator of reducing activity by several bacterial species, including *M. tuberculosis.*

We find that the formazans formed by reduction both of triphenyltetrazolium chloride and of neotetrazolium color selectively and accumulate around the granules of mycobacteria. *M. thamnopheos* reduced tetrazolium in concentrations in agar of 0.005 to 0.4 per cent and neotetrazolium in concentrations of 0.00001 to 0.1 per cent. Growth was inhibited with increasing concentrations and ceased at about 0.1 per cent concentration of both indicators. With tetrazolium at a concentration in agar of 0.005 per cent the granules appeared red in approximately 2 hours in *M. thamnopheos*, in approximately 72 hours in *M.*

[10] Mayer's albumin fixative as described on p. 620, McClung, C. E., "Handbook of Microscopical Technique," Paul B. Hoeber, Med. Book Dept. of Harper and Brothers, New York, New York, 2nd edition, April 1937.

[11] Fast Green National FCF, certified; dye content 91 per cent, cert. #Ngf-4; Arthur H. Thomas Company, Philadelphia, Pennsylvania.



FIGS. 1–15.

Figure 1. Mycobacterium thamnopheos, grown 36 hours at room temperature on 5 per cent glycerol agar containing 0.00001 per cent neotetrazolium.

Magnification on original negatives, figures 1 to 15 inclusive and A and B, 2,425 ×

Figure 2. Mycobacterium thamnopheos, same as figure 1. Counterstained with carbolfuchsin diluted 1:4 with distilled water for 2 minutes at room temperature. The densities seen in the centers of some cells as indicated by the arrow are due to the red of the carbolfuchsin and not the blue granules of neotetrazolium.

Figure 3. Mycobacterium thamnopheos, living cells on 5 per cent glycerol agar containing 0.1 per cent neotetrazolium and spread in a very thin film on a slide. The cells were under continuous observation for 4 hours and 43 minutes, during which time the polar granules gradually became dark blue due to the reduction of the neotetrazolium. Note the paired polar granules in the cell in the upper left-hand corner.

tuberculosis. With similar concentrations of neotetrazolium in agar the granules appeared bluish-purple in *M. thamnopheos* in less than 2 hours, and in virulent human cells in 23 hours. However, in cells under continuous observation in wet mounts, detectable reduction of neotetrazolium occurred in approximately 5 and 15 minutes for *M. thamnopheos* and *M. tuberculosis* var. *hominis*, strain B.V., respectively.

In all the organisms studied the sites of reduction of tetrazolium and neotetrazolium were similar in all aspects. The reduction areas first appeared as round granules. As the reduction continued the granules appeared to enlarge until their diameters were the same as that of the cell. Then they became egg-shaped with the smaller end pointed down the axis of the cell toward the center. The granules vary greatly in size from quite large ones to those that are barely discernible microscopically. Examples of the variation in shape and size may be seen in figures 1 to 11. Increase in size of the granules or change in shape seemed to take place by a process of building up of the colored compound around the original loci. In these loci of reduction the first color seen gradually intensifies until it reaches a maximum before the building up process begins. In general the large granules appear at the poles of the cell where they may be paired (see cell in upper left-hand corner of figure 3, also cells in figures 9 and 10). Occasionally large granules are centrally located, but usually the smaller granules are central (figures 1, 3, 5, 7, and 8). There are usually 2 to 4 granules per cell. In a given time interval not all of the cells reduce the tetrazolium or neotetrazolium, nor is the quantity reduced uniformly from cell to cell. In cells under continuous observation the colored reduced compound appears only in these granular areas.

Figure 4. Mycobacterium tuberculosis var. *hominis*, strain H37Rv, grown 120 hours at 37 C on 5 per cent glycerol agar containing 0.005 per cent neotetrazolium. Counterstained with carbol-fuchsin for 10 minutes at room temperature.

Figure 5. Mycobacterium tuberculosis var. *hominis*, strain B.V., same as figure 4 except grown for 168 hours.

Figures 6 and 7. Mycobacterium tuberculosis var. *hominis*, strain J.C., same as figure 4 except grown for 69 hours.

Figures 8, 9, 10, and 11. Mycobacterium tuberculosis var. *hominis*, strain P.T., same as figure 4 except grown for 96 hours. In figure 8 note that one cell is almost completely filled with reduced neotetrazolium which has gradually built up from particulate loci.

Figure 12. Mycobacterium thamnopheos, 5 drops of both 1 per cent α-naphthol in 95 per cent alcohol and 1 per cent dimethyl-p-phenylenediamine in water were added to 1 ml of a suspension of cells and allowed to stand for 2 hours, 24 minutes. A drop of the suspension was placed on an agar block and allowed to dry, then fixed for two minutes in vapours of 2 per cent OsO_4 solution. The dark polar granules were produced by the Nadi reagent and the darkening of the entire cell by the OsO_4 fixation acting on the Nadi reagent adsorbed by the cell (see footnote 7).

Figures 13, 14, and 15. Mycobacterium tuberculosis var. *hominis*, strain H37Rv. Same as figure 12 except: (a) cells were in Nadi reagent 23 hours; (b) cells in figure 13 were not fixed with OsO_4 vapours. At arrow in figure 13 the lower polar granule of the vertical cell is out of focus and is therefore hardly seen.

Figures A and B. Mycobacterium tuberculosis var. *hominis*, strain H37Rv. An impression smear stained by Harman's method for the selective staining of mitochondria except the cells were not counterstained with safranin.

Although the granular reduction areas were readily visible in permanent mounts of cells grown on tetrazolium and neotetrazolium, the remainder of the unstained cell was not visible (figure 1). By counterstaining cells grown on neotetrazolium-containing agar with carbol-fuchsin, the relationship of the granules to the entire cell was easily seen. The cells in figures 2 and 4 to 11 inclusive have been thus counterstained. Cells counterstained in this fashion were not uniformly light red, but frequently showed much darker red centrally located areas, as indicated by the arrow in figure 2; the significance of these intergranular bodies is under further investigation. They stain as nuclei by the De Lamater (1951) procedure.

Nadi reaction. The Nadi reaction is a specific indicator for the presence of cytochrome oxidase. Dimethyl-p-phenylenediamine is oxidized by cytochrome oxidase, in the presence of molecular oxygen and cytochrome c. If α-naphthol is present, a blue substance, indophenol blue, is formed (Hawk, Oser, and Summerson, 1947). As may be seen by comparing figures 12 to 15 with figures 1 to 11, the Nadi positive areas are also granules having the same distribution, size, and shape as the tetrazolium and neotetrazolium reducing areas. The Nadi positive areas appear within an hour after the Nadi reagent is added to the cell suspension. They do not change except for a slight increase in intensity after 23 hours.

Janus green B. According to Michaelis (1900) the safranin azo-dyes have in their molecules two chromophore groups, the azonium group characteristic of safranin and the azo group. Diethylsafraninazodimethylanilin (Janus green B) is dark green; when reduced with zinc dust it becomes first red, then colorless. "The first reaction indicates the breaking of the azo group, the second the reduction of the azonium group .... The same process takes place in the organism." Extensive application of Janus green B by various investigators, notably E. V. Cowdry (1918, 1924), has confirmed its specificity when used in high dilutions as a mitochondrial stain.

In mycobacteria grown on agar containing Janus green B only the first stage of this specific mitochondrial staining reaction occurred. That is, polar granules in the cells were stained green while the remainder of the cell was colorless. These polar granules are in every aspect like the tetrazolium and neotetrazolium reducing granules and the Nadi positive granules. Indeed when tetrazolium was included in the agar with Janus green B, the granules were shown to be the same. The cells showed reddish-green granules; none had separate red and green granules.

By adding Janus green B to cell suspensions it was also possible to elicit the reaction. First the granules stained green, then became red, and finally this color faded. When the cells had settled to the bottom of a test tube after standing for a time, all stages of the reaction could be seen. The edge of the sediment was green, then there was a red band, and finally a white central area of cells. The color changes were also seen to occur in the granules of cells in a Janus green B solution which was sealed with melted paraffin on a microscopic slide under a cover slip.

Acid-hematin stain. The staining of phospholipids with acid-hematin according to the procedure described by Baker depends on mordanting of the phospholipids with dichromate which then will combine with the acid-hematin. Differentiation is subsequently done with a borax-ferricyanide solution; the phospholipids and certain proteins stain a dark blue or blue-black. Differentiation between these two components is achieved by first removing the phospholipids with a pyridine-extraction method and then mordanting with dichromate-calcium and staining with acid-hematin. A positive test for phospholipids is indicated when areas or structures stained blue or blue-black following the acid-hematin stain are no longer colored in this manner following the pyridine-extraction method. If the areas or structures are still dark blue or blue-black following pyridine extraction, then they are not phospholipids. Mitochondria give the acid-hematin stain, but mitochondria are not stained after pyridine extraction (Baker, 1946).

After being stained with the acid-hematin stain the mycobacterial cells in all cases were blue-black throughout. However, with careful observation and the use of colored filters, one could see that at the poles, and occasionally in the center there were round areas of increased density. These areas were of the same size and distribution as the granules previously described. Following application of pyridine extraction and then acid-hematin staining neither the blue-black of the cells nor of the dense areas could be seen. The cells simply appeared as slightly gray refractile areas.

Harman's stain. Harman's stain utilizes a sulfonated, acid, triphenylmethane dye dissolved in a suitable solvent—aniline water, and gentle heating. These empirical criteria for the selective staining of mitochondria were derived from the staining procedures of Altmann, Benda, Regaud, and modifications of these.

Our preparations stained by this method showed bright green cells while at the poles and occasionally in the center were denser bluish-green granules. The contrast between the granules and remainder of the cell was not marked but with proper filters it could be brought out sharply. The granules were round, discrete polar bodies. Occasionally, they were paired and a few cells had an additional granule or two centrally located. They were similar to the granules previously described.

Electron microscopic observations. Undisturbed microcolonies of *M. thamnopheos* are shown in figures 16 to 22. The cells of the strain parasitic for poikilothermic animals, which is avirulent for mammals (Aronson, 1929), lie side by side or end to end.

The mycobacterial granules, some of whose enzymatic activities have been described, are most clearly shown in electron micrographs (figures 16 to 22). They are seen to be exceedingly electron-opaque objects, round or elliptical in optical section, and with smooth contours. The contours of the granules have minimal areas (cf. Plateau, 1864), except where deformed by the contiguous cytoplasmic vacuoles (figures 18 and 19). Deformation of the granules by the cytoplasmic vacuoles has been particularly emphasized by Knaysi, Hillier, and Fabricant (1950) whose pictures of *M. tuberculosis* var. *avium* bring out the

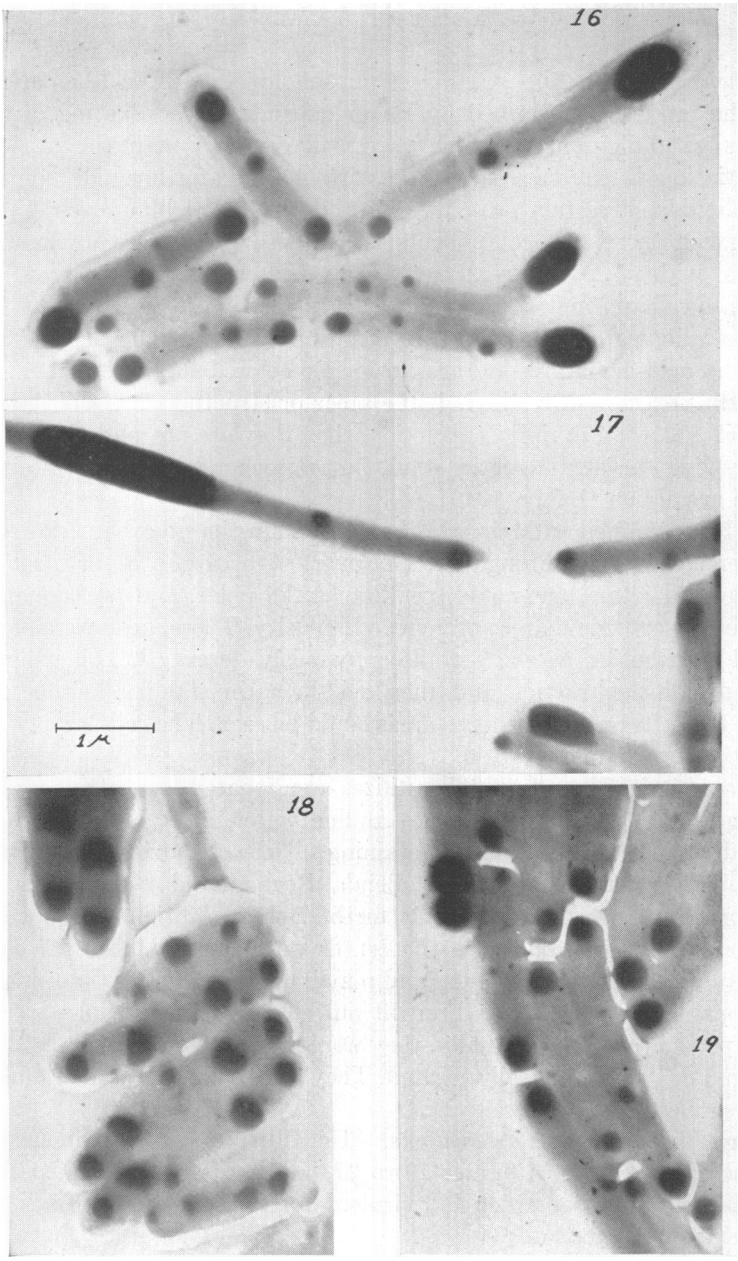


Figure 16. Mycobacterium thamnopheos grown 26 hours at room temperature on a collodion film on the surface of 5 per cent glycerol agar (extract broth base). Specimen was mounted on copper screen and dried in a desiccator over $CaCl_2$ for 24 hours prior to examination.

Magnification on original negatives, figures 16 to 22 inclusive, 5,800 ×

Figure 17. Mycobacterium thamnopheos, same as figure 16 except the specimen was treated for 1 minute in 2 per cent OsO_4 vapours before being mounted and dried.

Figure 18. Mycobacterium thamnopheos, same as figure 16 except it was grown for 6 hours only.

Figure 19. Mycobacterium thamnopheos, same as figure 16.

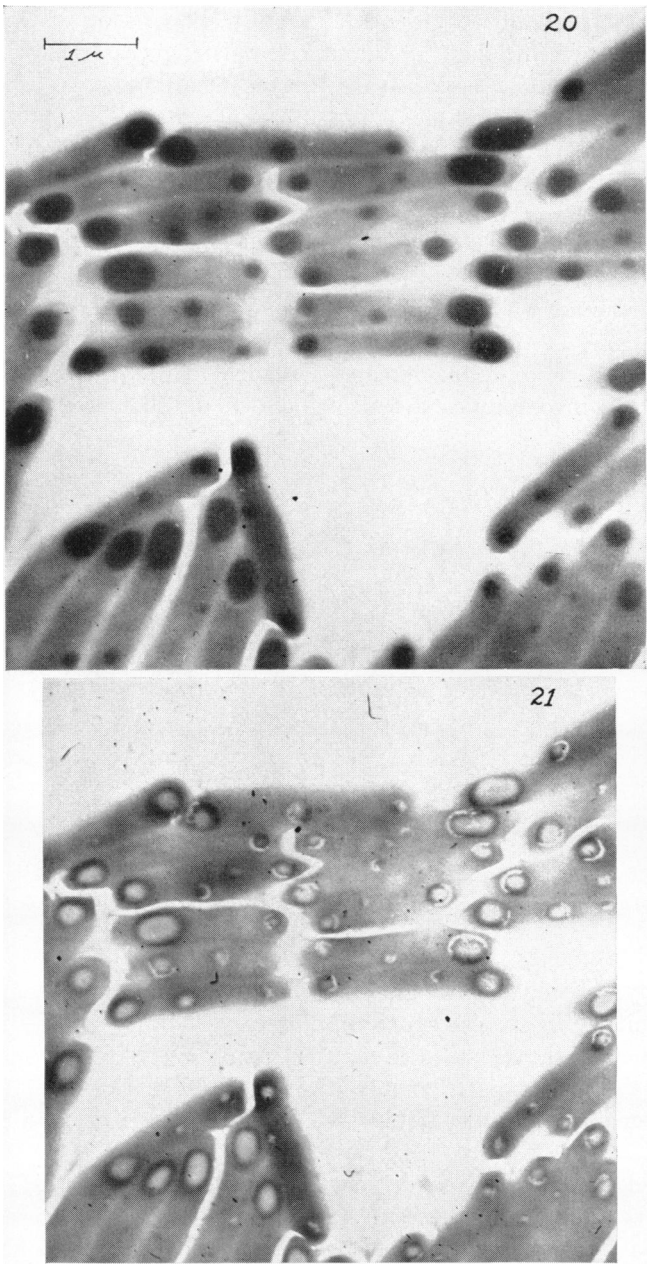


Figure 20. *Mycobacterium thamnopheos*, same as figure 16 except grown for 48 hours.
Figure 21. Same specimen and field as figure 20 following intensive electron bombardment.

vacuolar system more clearly than the pictures presented here. The dark granules are obviously within the cytoplasm. The cytoplasm is characteristically

shrunken away from the very delicate cell-wall. Separation of the protoplasts of adjacent cells within the still intact cell-wall (figures 16 and 17) suggests beginning cell division as described by Knaysi (1929).

The electron opaque granules vary in form from relatively large prolate ellipsoids to tiny spheres approaching the limit of definition in the electron pictures. The largest granule in figure 16 is 0.75 μ by 0.5 μ, the largest in figure 17 is 2.0 μ by 0.48 μ. These dimensions are within the range of dimensions of mitochondria of animal cells as determined by Claude (1947–48) and co-workers (Claude and Fullam, 1945, 1946). Zollinger (1948) gives the dimensions of mitochondria in fresh cells of the frog kidney and small intestine, viewed with the phase microscope, as 0.1 μ wide by 0.8 to 1.2 μ long. Cf. also Fraunsteiner (1951) and Hogeboom, Schneider and Pallade (1948). The smallest electron opaque granules in our mycobacteria which can be identified with confidence

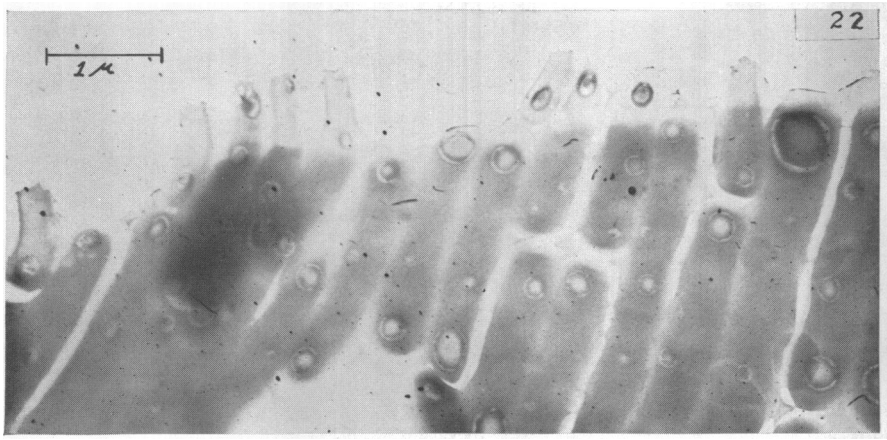


Figure 22. Mycobacterium thamnopheos, same as figure 21, demonstrating the "fixation" phenomenon.

with the enzymatically active granules measure about 0.07 μ, and are thus below the limits of light microscopic resolution.

The tendency of the granules to occur in pairs (cf. figures 18 and 19) is suggestive that the larger particles may divide (cf. E. V. Cowdry, 1924; Wilson, 1925). The smallest granules could well be synthesized *de novo* in the cytoplasm.

When the electron beam is made very intense, the granules of *M. thamnopheos* may be observed on the fluorescent screen of the electron microscope to volatilize explosively. Torn and distorted membranes may be seen in figures 21 and 22 surrounding the residues of the granules. More difficulty was experienced in volatilizing the granules of *M. tuberculosis*, but these, too, showed vacuolated centers after prolonged and intense electron bombardment. Volatilization of the granules in mycobacteria by intense electron bombardment has previously been demonstrated by Lembke and Ruska (1940) and by Knaysi, Hillier, and Fabricant (1950).

The "fixation" phenomenon previously described by Hillier, Mudd, Smith,

and Beutner (1950) is illustrated in figure 22. An electron picture was taken of the field shown, the field was then subjected to intense electron bombardment by increasing the intensity of the beam, and finally a second picture was taken. The specimen must have moved slightly between the first picture and the intense bombardment so that the upper part of the specimen, which had been protected from irradiation in taking the first picture, was subjected to the subsequent intense bombardment. It may be seen that the upper, previously unirradiated portion of the specimen was almost completely volatilized by the intense irradiation. The cytoplasm of the lower part of the specimen on the contrary had been "fixed" by the irradiation used in taking the first picture. The mitochondrial granules were not equally protected by electronic fixation and were for the most part volatilized in both parts of the specimen.

Electron irradiation has previously been shown (von Borries and Glaser, 1944; König and Winkler, 1948; Hillier, Mudd, Smith, and Beutner, 1950) to "fix" bacterial cytoplasm against subsequent action by heat and by lytic agents. This fixation is shown here to make the cytoplasm refractory also to subsequent volatilization by intense electron irradiation.

Incidental observations. In cells of all the mycobacterial strains studied, stained 30 seconds in aged Loeffler's alkaline methylene blue diluted 1:8 with distilled water, the granules appeared bright red, with the rest of the cell blue. This effect is attributed to staining of the granules with methylene violet present in the aged methylene blue (Knaysi, 1951; refer to p. 267). In cells stained 5 minutes in 0.3 per cent Sudan black B in 95 per cent alcohol at room temperature the granules were dark blue-black and the cells were light blue. The granules in the mycobacterial cells stained by Hucker's modification of the gram stain were strongly gram-positive.

DISCUSSION

The already voluminous literature on mitochondria was reviewed comprehensively by E. V. Cowdry in 1918. He wrote:

"Mitochondria have been carefully studied in phylogeny as well as in ontogeny. Their wide distribution is amazing. It has already been pointed out that they occur from man to the most lowly protozoon and from the angiosperms to the fungi, though their existence is doubtful in the myxomycetes, schizomycetes, and most of the algae. They are apparently identical in both plants and animals . . . . They are indeed as characteristic of the cytoplasm as chromatin is of the nucleus."

E. V. Cowdry (1918) states at another place in the same monograph: "I have found that large bacilli contain granules which stain intensely and apparently specifically with Janus green. They resemble in distribution the so-called polar granules. Smaller forms often stain diffusely."

These observations seem not to have been followed up (E. V. Cowdry, 1951). E. V. Cowdry in 1924 again wrote of mitochondria, "their existence is doubtful in bacteria." See also Knaysi (1951). N. H. Cowdry (1918) reported mitochondria in myxomycetes.

In the intervening years many investigations have been focused on the iso-

lation of mitochondria free from cells and on the coordinated systems of enzymatic reactions mediated by mitochondria. This literature has been reviewed recently and well by Dounce (1950), Bradfield (1950), Green (1951), Lehninger (1951), and Schneider and Hogeboom (1951). The morphology of mitochondria has also been studied more closely, in particular with the aid of the electron microscope (Claude, 1947–48; Claude, 1950; Dalton, Kahler, Kelly, Lloyd, and Striebich, 1949; Mühlethaler, Müller, and Zollinger, 1950), and phase contrast microscope (Zollinger, 1948, 1950; Harman, 1950*b*,*c*).

In view of the steady accumulation of evidence that bacteria are cellular organisms possessing the fundamental attributes common to all organisms, it is amazing that the question of the existence or nonexistence of mitochondria in bacteria should have been so singularly neglected. Alexeieff (1924) did interpret certain bacterial granules which stained with iron hematoxylin as mitochondria.[12] And one short note does explicitly state that the granules in *M. tuberculosis* are not mitochondria (Hollande and Crémieux, 1928), on the basis of what we can only regard as faulty observations.

Our procedure in this study has been to examine the granules of mycobacterial strains intracellularly in as many reactions as may afford definitive comparison with the known attributes of mitochondria. A more exhaustive and possibly more convincing comparison may be made if the bacterial granules can be isolated intact and subjected to systematic examination for enzymatic activities. Work to this end is underway.

Dounce (1950) concludes: "Evidence has been presented which decisively demonstrates the occurrence of many enzymes in small cell particles, such as nucleoli, chromosomes, nuclei and mitochondria, and on cell surfaces. In some cases (cytochrome oxidase and succinic dehydrogenase) it seems clear that none of the enzyme occurs dissolved in the cytoplasmic matrix, and in the case of succinic dehydrogenase at least, all of the enzyme seems to be located within the mitochondria. The bulk of the cytochrome oxidase and cytochrome system is also located in the mitochondria, and in addition, it now seems probable that mitochondria carry the entire system of enzymes participating in the Krebs cycle for the oxidation of carbohydrate. These particles also carry enzymes necessary for the oxidation of fatty acids." Cf. also Harman (1950).

Schneider and Hogeboom (1951) state: "Cytochrome c, vitamin B_6, and riboflavin are concentrated in mitochondria to a considerable extent, the latter finding indicating the probable presence of flavoproteins not as yet studied."

Observations presented here demonstrate the localization of the reduction of tetrazolium, probably mediated by the flavoprotein dehydrogenase diaphorase, and of cytochrome oxidase in the granules of mycobacteria. These granules also give the color reactions to Janus green B, acid-hematin, and to Harman's stain which are characteristic of mitochondria.

[12] The author's comment on these observations (Alexeieff, 1924) was: "dans la deuxième partie je me suis occupé de l'étude des mitochondries et des mucosomes; les résultats de ces recherches sont loin de présenter le même caractère de certitude et ne peuvent servir que comme hypothèses de travail."

Morphologically the mycobacterial granules have the appearance, size, and smooth contours of minimal areas (E. V. Cowdry, 1924) characteristic of mitochondria. Like mitochondria of other cells (cf. Claude and Fullam, 1945, 1946; Dalton, Kahler, Kelly, Lloyd, and Striebich, 1949; Mühlethaler, Müller, and Zollinger, 1950), they have limiting membranes visible in electron micrographs.

In view of the many points of similarity between the respiration of bacteria and of the cells of larger forms, and of the known coordination within mitochondria of the cytochrome system and of the tricarboxylic acid cycle, it seems surprising that the occurrence of mitochondria in bacterial cells should have gone unrecognized for so long. In a following paper we shall present evidence of the occurrence of cytoplasmic particles with similar enzymatic and tinctorial properties in other bacterial genera.

SUMMARY

The granules of mycobacteria, interpreted by Koch as spores and variously by other investigators, are here shown to be centers of oxidative-reductive enzymatic activity. Triphenyltetrazolium chloride and neotetrazolium are reduced to their colored formazans in these mycobacterial granules. Such reduction has been shown to be catalyzed by the flavoprotein dehydrogenase diaphorase. The Nadi reaction for cytochrome oxidase is positive also in these granules.

The mycobacterial granules stain initially blue-green with Janus green B; reduction of this specific mitochondrial stain in the granules changes their color to red which finally fades. The granules give the Baker stain for phospholipid and the Harman mitochondrial stain.

Morphologically the mycobacterial granules resemble the mitochondria of animal cells in size, contour, and plasticity. When the granules are volatilized in the electron microscope by intense electron bombardment, wrinkled and torn surface membranes remain.

Since the granules of mycobacteria are shown to resemble mitochondria in all enzymatic, tinctorial, and morphological attributes in which they have been compared, we conclude that they are mitochondria. This we believe to be the first definitive recognition of mitochondria in bacteria. Bacteria in another aspect are demonstrated to have fundamental attributes in common with other organisms.

ADDENDUM: A paper by Georgi, C. E., Militzer, W., Burns, L. and Heotis, J. (1951 On the existence of a cell granule in a thermophilic bacterium, Proc. Soc. Exptl. Biol. Med., **76,** 598–601), describing separation of enzymatically active granules from a thermophile, came to our attention after this manuscript was submitted for publication.

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