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Ultrastructure of *Mycobacterium lepraemurium* [1, 2]

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In a study on the *in vitro* culture of *Mycobacterium lepraemurium* one of the most common observations was the occurrence of two types of inclusions within the cells. These were present both in organisms freshly isolated from lepromas in rats and mice and also in organisms kept under certain cultural conditions for several weeks. Information on the effects of different cultural conditions on the organisms will be described in a later paper. The present paper is a report of observations on the ultrastructure of organisms isolated from lepromas in rats and mice. In one case (Fig. 2), for convenience, a photograph of an organism kept *in vitro* is used.

MATERIALS AND METHODS

M. lepraemurium (Hawaiian strain) was inoculated subcutaneously into the groins of albino mice. After about 15 weeks the lepromas were harvested and ground aseptically in a mortar in 1% albumen-saline ([19]). The suspension was then centrifuged lightly (600 rpm for 5 min.) to remove large particle debris and the supernatant was centrifuged again at 3,000 rpm for 10 minutes to sediment the organisms. The resuspended organisms were used as inoculum for various media to be tested, or for re-inoculating animals, or were used for electron microscopic observation.

Mycobacterium leprae. Cells of *M. leprae* were obtained from a punch biopsy of a wrist lesion of a patient with lepromatous leprosy. The patient had not received any treatment. The tissue was macerated and bacterial cells obtained by the method described above. Negatively-stained preparations were examined in a Siemens electron microscope.

Electron microscopy. (a) *Negative-staining.* One ml samples of the suspension were centrifuged and the supernatant fluid removed. Two drops of 2% phospho-tungstic acid adjusted to pH 7.0 with KOH were then added to the pellet and the organisms shaken to suspend them. They were then stored at 4°C until examined. One drop was placed on a Formvar-coated grid, left for a few seconds and then the liquid was absorbed off with filter paper. Grids were examined with either a Phillips EM200 or a Siemens Elmiskop Ia electron microscope. Photographs were taken in the former on 35 mm. film and in the latter on Ilford 6.5 x 9.0 cm. glass plates. Development for the film was in Kodak D-19 and for the glass plates in D-76 (b) *Sections.* Organisms were fixed in 1% (w/v) osmium tetroxide in acetate-veronal buffer, pH 6.1, following the procedure of Kellenberger *et al.* ([11]). Ten per cent (v/v) of 1% OsO_4 was added to a suspension of the organisms in albumen-saline and the organisms sedimented by centrifugation. The pellet was then resuspended in 1 ml OsO_4 solution and 0.1 ml tryptone medium and left in the dark overnight at room temperature. After diluting with acetate-veronal buffer the organisms were sedimented and suspended in a small volume of 2% Noble agar at 45°C which, after hardening, was cut into small cubes. These cubes were then treated with 2% uranyl acetate for two hours and then dehydrated in an acetone series and, departing from the procedure of Kellenberger *et al* ([11]), taken into two changes of propylene oxide for 30 minutes each. The embedding procedure was based on that of Luft ([15]). After the propylene oxide treatment, the blocks were placed in a 1:1 mixture of propylene oxide:complete Epon mixture and left overnight uncovered. The

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Epon mixture used was a ratio of 3:7 mixture A:mixture B with 1.5% DMP-30 (the accelerator) added. The blocks were then transferred to the surface of fresh Epon mixture (without propylene oxide) and allowed to sink while gelatin or Beem capsules were being prepared by adding one drop of Epon to each capsule and labelling. The blocks were transferred to absorbent paper with Pasteur pipettes and then transferred with a minimum of Epon to the Epon in the capsules. The capsules were then filled with fresh Epon. After degassing in a vacuum desiccator the blocks were cured for one day at 35°C, one day at 45°C and several days at 60°C. Sections were made with a diamond knife and mounted on Formvar-coated copper grids. They were stained for 4 to 6 minutes with uranyl acetate ([27]) and 4 to 6 minutes with lead citrate ([6]).

RESULTS

The most striking feature observed was the presence of electron-dense particles present in the presumed healthy (unshrivelled) cells (Fig. 1). The particles or inclusions had an average diameter of about 30 mμ with a range of about 10 mμ to 60 mμ. They were not uniformly distributed throughout the cells but occurred generally in discrete clusters with several clusters to a cell. The clusters were arranged around parts of the cells which were not so electron-dense as the inclusions themselves but which were most often slightly more electron-dense than the rest of the cell contents when the cells were negatively-stained. These larger bodies of low electron-density represent a second significant inclusion. The larger bodies appeared more solid than the rest of the cytoplasm or at least were largely impermeable to the smaller electron-dense particles. The solid nature was demonstrated in photographs in which the cells were shrivelled and the cytoplasm contracted between the large solid bodies (Fig. 2). The smaller particles appeared to be present in the cytoplasm and were arranged between the larger bodies (Figs. 1 & 2). If the larger bodies were close togeth-

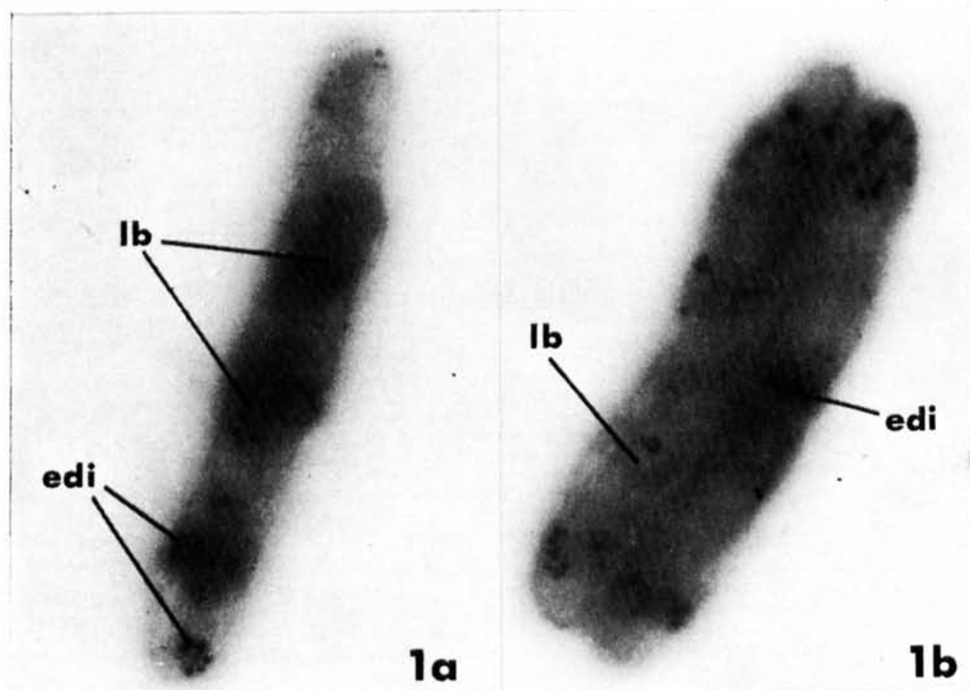


FIG. 1. Negatively-stained (PTA) preparations of *M. lepraemurium* showing small electron-dense inclusions (**edi**) and larger bodies (**lb**) of lower electron-density. Magnification: a. × 30,000, b. × 30,000.

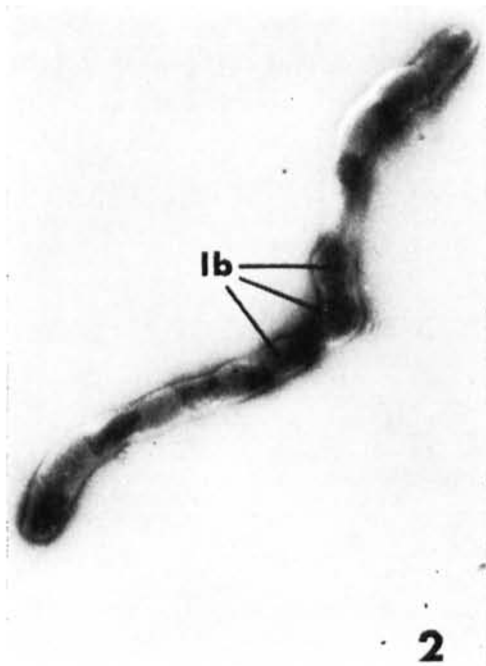


FIG. 2. Negatively-stained (PTA) preparation of *M. lepraemurium* illustrating the solid nature of the larger bodies (**lb**). Organism kept in culture medium for three weeks at 37°C. Magnification: × 10,500.

er the smaller particles were pushed against the larger ones so that smaller particles outlined the peripheries of the larger bodies in some photographs. The larger bodies had an average diameter of about 175 mμ with a range of about 120 to 300 mμ. Both types of inclusions were demonstrable in unstained preparations as well as in preparations negatively-stained with PTA. In some photographs and especially in those taken at higher primary magnifications the electron-dense inclusions appeared to be composed of several globules with electron-transparent centers and electron-dense borders (Fig. 3).

The smaller electron-dense particles were also observed in the few cells of *M. leprae* examined (Fig. 4) and vague outlines of the larger bodies may be discerned in some photographs.

In sections of *M. lepraemurium* the two types of inclusions observed in negatively-stained preparations could be clearly seen (Figs. 5 & 6). After staining with uranyl acetate and lead citrate the larger bodies appeared more electron transparent than the rest of the cytoplasm. These bodies were homogeneous, finely granular masses without sharply defined edges (Figs. 5 & 7). The edges of the smaller electron-dense particles were better defined and appeared to be surrounded by membranes (Fig. 8). In some photographs of sections the smaller particles were seen to be embedded in the larger bodies (Fig. 5).

In addition to the inclusions many cells had internal double membranous structures (Fig. 9). These rarely appeared to be organized into typical mesosomes. A structure which may be a mesosome is shown in Figure 10. In many photographs the internal membranes were seen to be continuous with the cell membrane (Figs. 5 & 11).

All three types of structure were distinct from the chromosomal regions of the cells

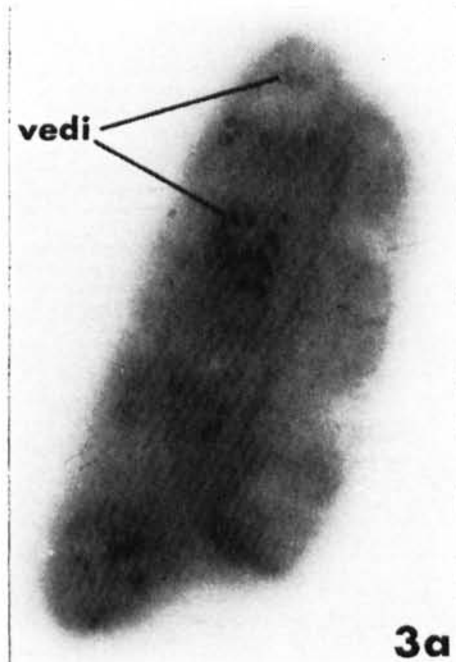


FIG. 3. Negatively-stained (PTA) preparations of *M. lepraemurium* showing the vacuolized electron-dense inclusions (**vedi**) after exposure to high intensity electron beams. Magnification: a. × 44,000. b. × 90,000. c. × 153,500.

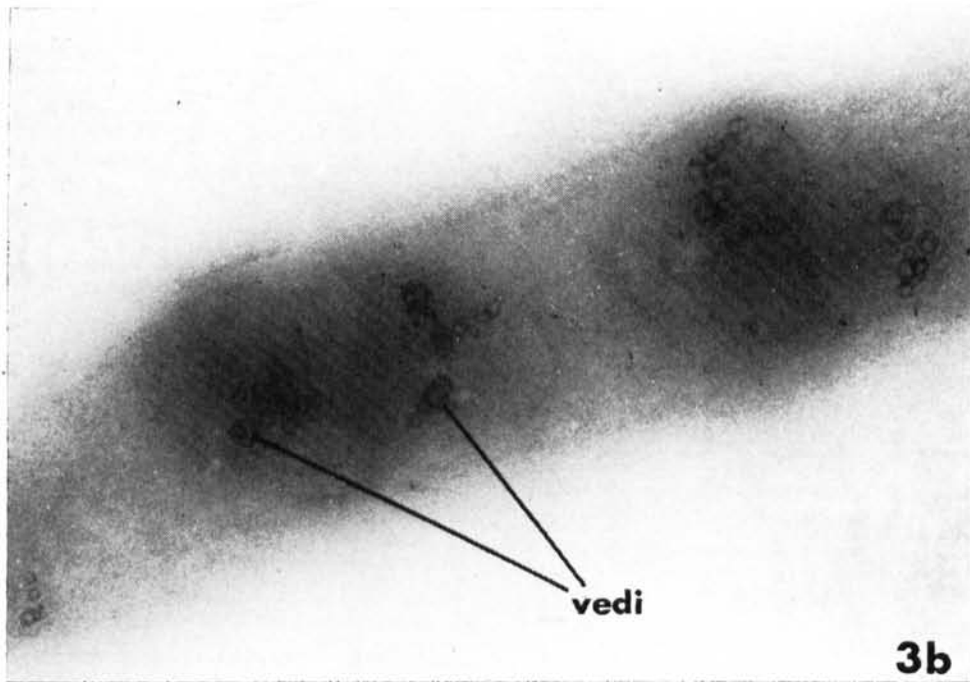


FIG. 3. Negatively-stained (PTA) preparations of *M. lepraemurium* showing the vacuolized electron-dense inclusions (**vedi**) after exposure to high intensity electron beams. Magnification: a. × 44,000. b. × 90,000. c. × 153,500.

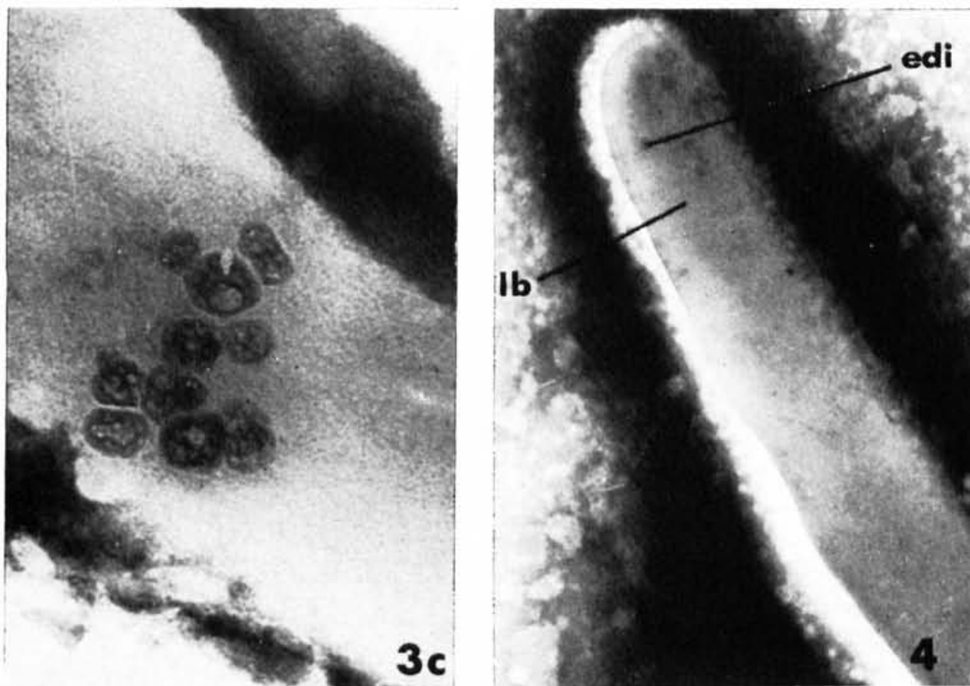


FIG. 4. Negatively-stained preparation of *M. leprae* showing electron-dense inclusions (**edi**) and larger bodies (**lb**). Magnification: × 53,000.

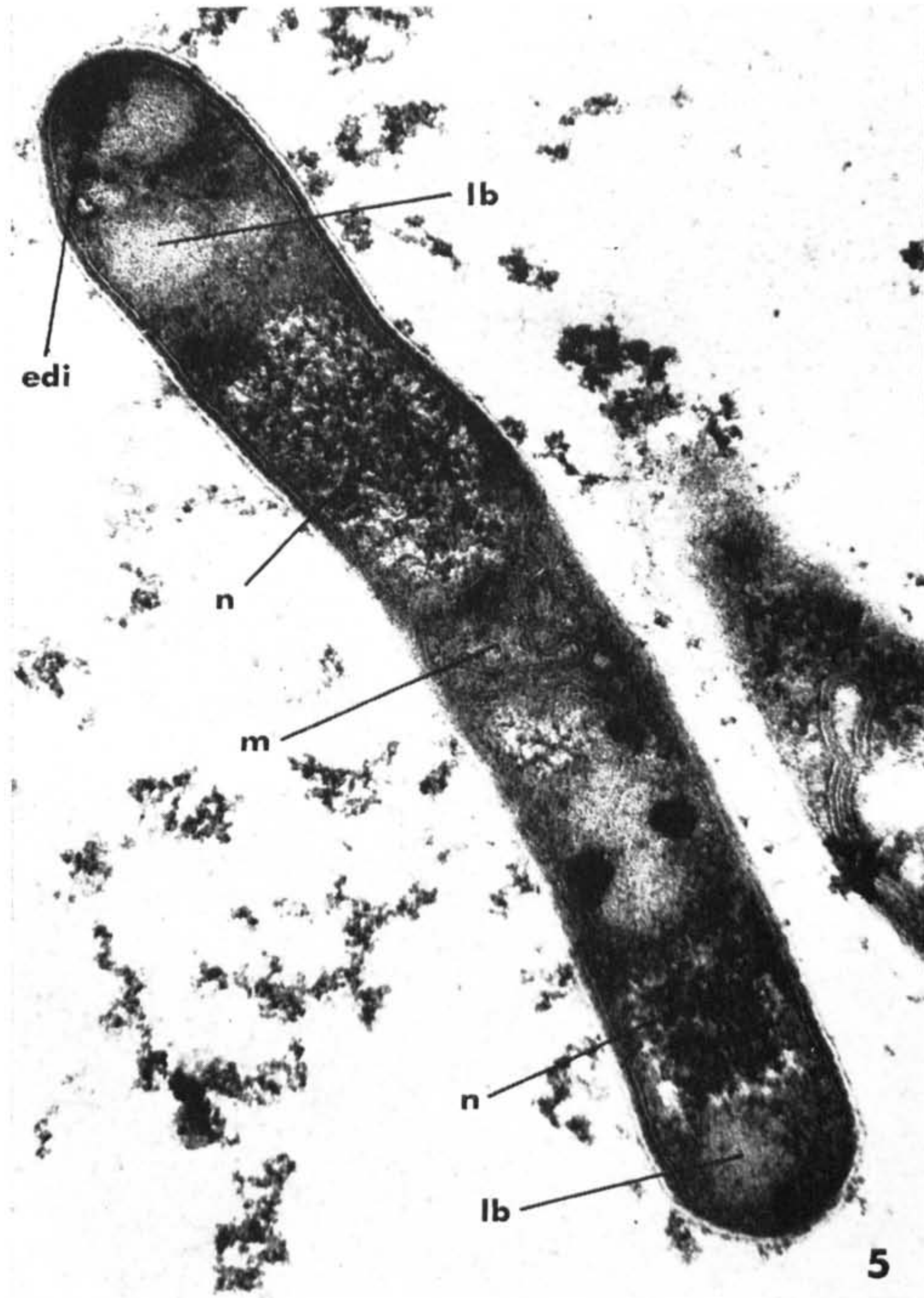


FIG. 5. Section of *M. lepraemurium* stained with uranyl acetate and lead citrate showing electron-dense inclusions (**edi**), larger bodies (**lb**), nuclear material (**n**) and internal structure (**m**). Magnification: × 78,000.

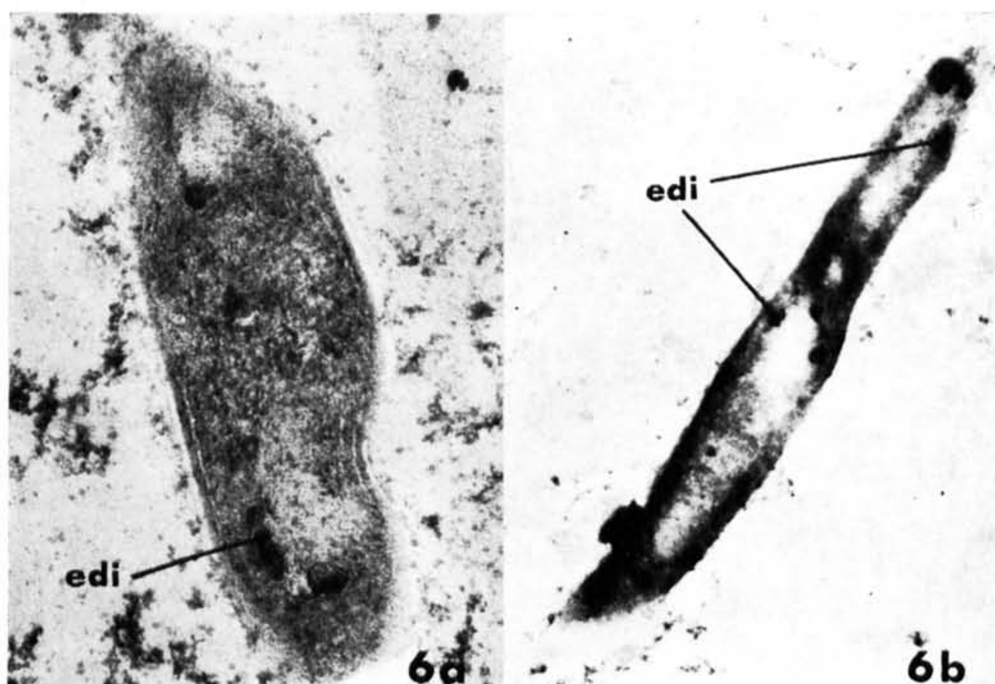


FIG. 6. Sections of *M. lepraemurium* stained with uranyl acetate and lead citrate illustrating the electron-dense inclusions (**edi**). Magnification: a. × 88,000, b. × 37,000.

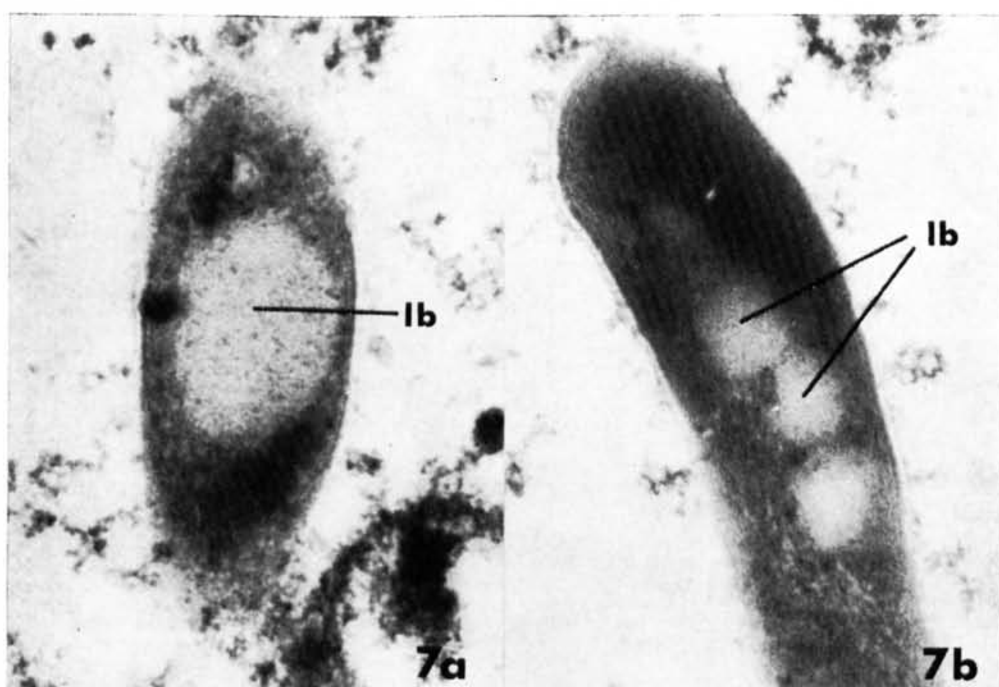


FIG. 7. Sections of *M. lepraemurium* stained with uranyl acetate and lead citrate showing the finely-granular, diffusely-bordered larger bodies (**lb**). Magnification: a. × 102,000, b. × 105,000.

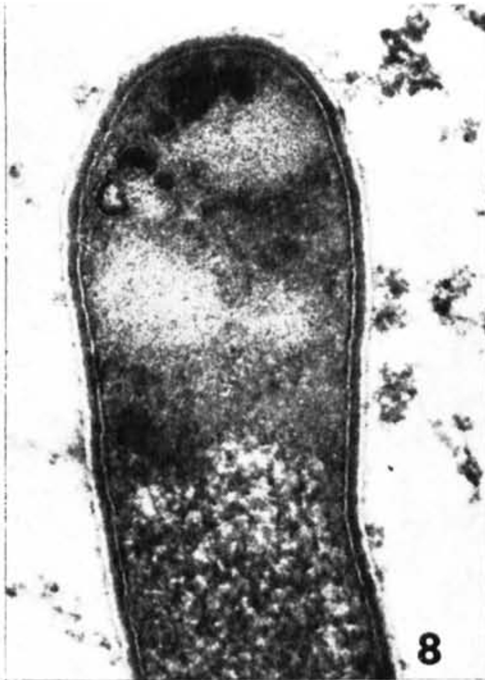


FIG. 8. Electron-microscopic enlargement of a portion of the organism shown in Figure 5 showing the apparent membranes around the electron-dense inclusions. Magnification: × 87,000.

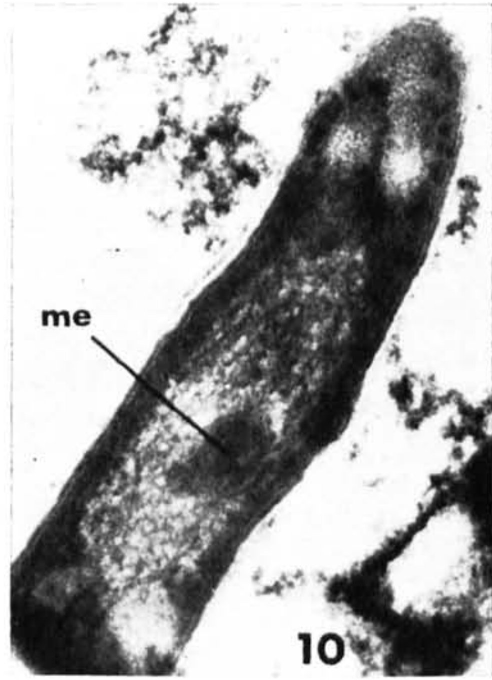
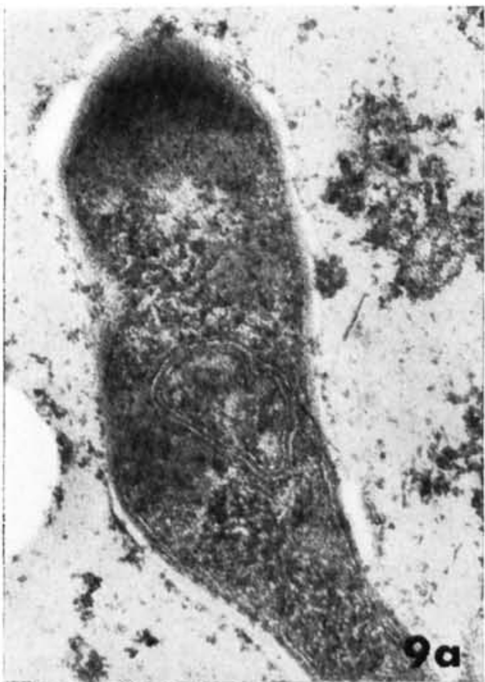


FIG. 10. Section of *M. lepraemurium* stained with uranyl acetate and lead citrate showing a lamellar-type mesosome (**me**) embedded in the chromosomal region of the cell. Magnification: × 87,000.



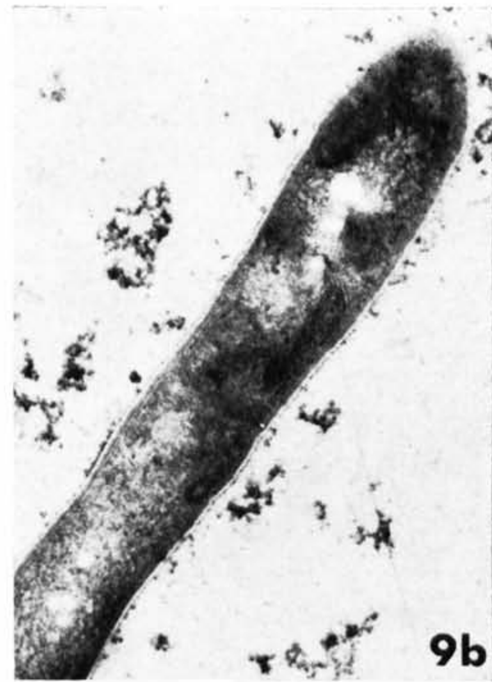


FIG. 9. Sections of *M. lepraemurium* stained with uranyl acetate and lead citrate showing internal membrane structures. Magnification: a. × 78,000, b. × 53,000.

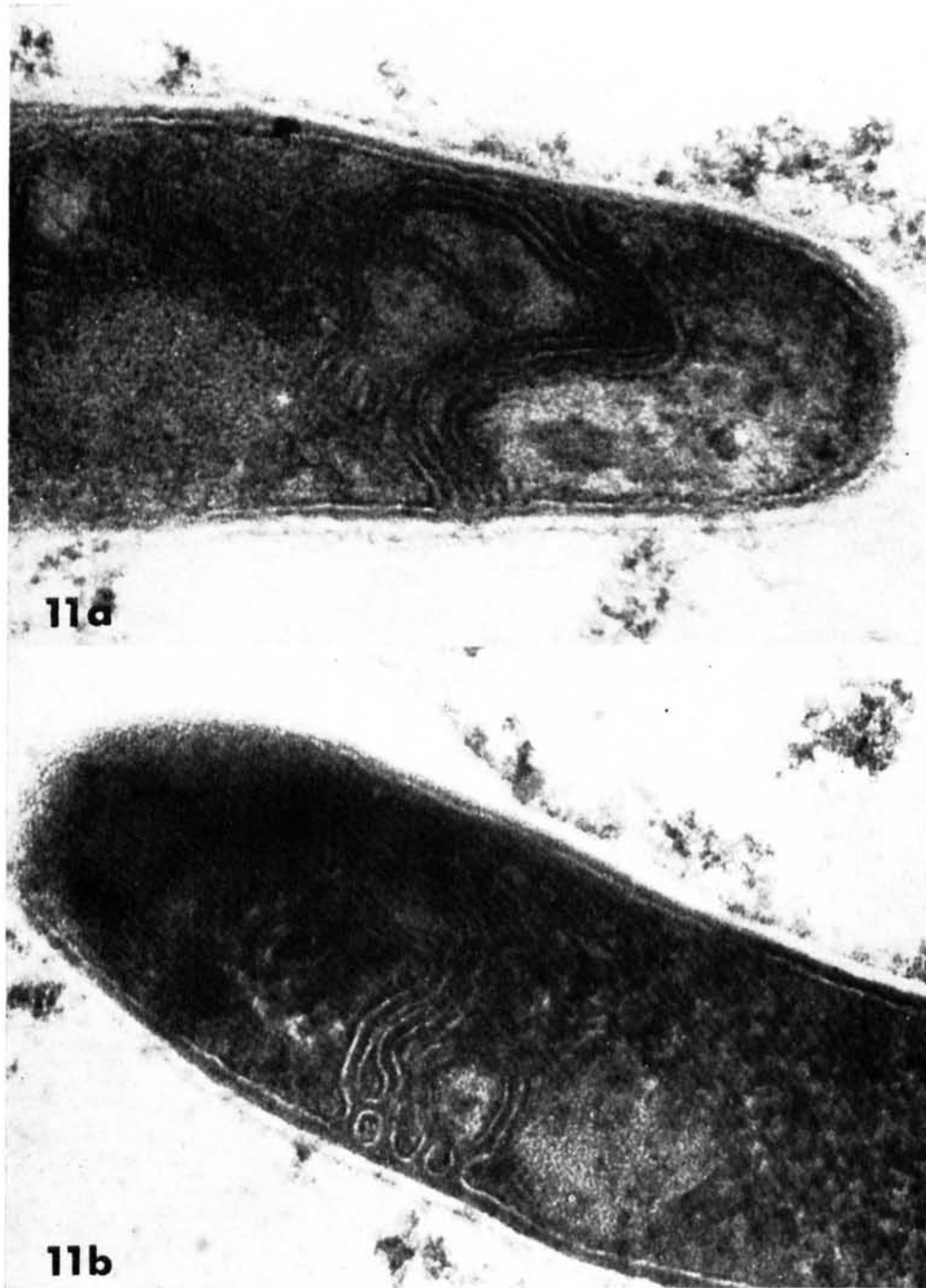


FIG. 11. Sections of *M. lepraemurium* stained with uranyl acetate and lead citrate showing internal membranous structures continuous with the cell membranes. Magnification: a. × 215,000, b. × 173,000.

which in sections (Figs. 5 & 8) had a coarsely-granular structure.

DISCUSSION

Electron-dense inclusions have been reported in several species of mycobacteria, e.g., *M. avium* [1, 2, 12]; *M. avium* (Jucho strain) [28, 29]; *M. johnei* [26]; *M. leprae* [9]; *M. phlei* [5, 8, 29]; *M. smegmatis* [7]; *M. thamnopheos* [18]; *M. tuberculosis* [2, 14, 18]; BCG [17, 21, 22, 23, 29, 30]. The electron-dense inclusions were shown to contain metaphosphate (volutin) by Glauert and Brieger [8] by a comparison of the same organisms of *M. phlei* stained for volutin and examined by both light and electron microscopy. Most of the typical electron-dense volutin deposits reported appear considerably larger than the electron-dense particles which we found in *M. lepraemurium*. Shinohara [21] found a range in size of electron-dense particles of up to 450 mμ diameter with a mode of 101-105 mμ for the three specimens of BCG he investigated. One of his three specimens, however, had a mode of 1 to 50 mμ and contained 31.6% of the granules investigated. The largest one we measured was about 60 mμ in diameter. They also correspond in size and appearance to the small phosphate granules shown in *M. tuberculosis* by Kölbel [14] and the "mikogranula" of Lembke and Ruska (1940) and Wessel (1942) as quoted by Mudd *et al* [17].

Knaysi [12] called the large electron-dense inclusions "A bodies" and the electron-transparent bodies "C bodies". To this Takeya *et al.* [29] added the small electron-dense granules (type B) which correspond to our electron-dense granules. They found type B granules in *M. phlei*, avian and human tuberculous bacilli and BCG, and these were present in circular groups of up to 20 granules. They seemed to increase in number, and the groups in size, during growth of the organisms and finally attained the size level of A granules. Shinohara [22] found a tendency for minute granules (less than 50 mμ diameter) to increase with age of culture, but he could not find any evidence of minute granules combining to form large granules or of production of small from large granules. He found that granules (presumably the usual larger granules) were not present in isolated cells of BCG which could not form microcolonies though they were frequently found in cells which could form microcolonies. Smith [26] also found two size groups of electron-dense bodies in *M. johnei*, the larger (70 to 180 mμ diameter) corresponding in size and distribution to volutin as it is most often seen, and smaller ones (10 to 20 mμ diameter) which a proportion of cells contained and which he suggests are small accumulations of volutin.

Mudd *et al.* [17] showed that when *M. thamnopheos* and BCG were grown in or on media containing suitable oxidizable substrates (malate or glycerol) larger metaphosphate deposits were produced. But in BCG cells depleted of metachromatic material by maintenance in the absence of a suitable oxidizable substrate (with glucose as the energy source or by incubation in dilute buffer or growth on a collodion film overlying egg medium) the metaphosphate occurred in the form of minute granules which are similar in appearance to those we found in *M. lepraemurium*. Imaeda and Convit [9] stated that, as neither the electron-dense granules nor intracytoplasmic membrane systems are found in degenerating *M. leprae* cells, these two components may represent indicators of the state of bacillary activity. Shinohara [22] found a marked increase in the proportion of small inclusions if the cells were kept in sodium chloride and distilled water or under other unfavorable conditions for a long time [23]. Shinohara and Fukushi [23] demonstrated granules within or around circumscribed areas of greater density than the surrounding cytoplasm, corresponding probably to our larger inclusions. The granules generally appeared in periods of active growth and decreased if the organisms were inhibited or kept under unfavourable conditions. Kölbel [14] was of the opinion that the granules in mycobacteria play an important role in the cell metabolism. Takeya *et al.* [29] found that in the initial period of growth the granules, if present, were small and the cell bodies generally not electron-transparent,

but as growth proceeded the cell bodies became transparent and granules appeared, but in still older cultures the granules decreased in numbers.

When the granules are exposed to a high intensity electron beam they become partially volatilized and appear vacuolated ([8, 17, 21, 28, 30]). The inclusions in our photographs taken at higher primary magnifications which require a higher intensity electron beam also have a vacuolized appearance (Fig. 3). The inclusions have more electron-transparent centers with distinctly more dense borders, suggesting, as Glauert and Brieger ([8]) point out, that they contain substances other than volatile metaphosphates. Contrary to the experience of Shinohara ([21]) we found no evidence of *de novo* production of particles by bombarding cytoplasm previously devoid of particles.

The smaller electron-dense inclusions which we found in *M. lepraemurium* are almost certainly granules containing metaphosphates similar to those found by others in other species of mycobacteria. Evidence for their content of inorganic material is given by their high electron-density since organic material (proteins, carbohydrates, lipids) are not as electron-dense. Their small size may be indicative of the low growth rate and metabolic ability of this particular organism, but their presence and extent may possibly be used as an index of the suitability of cultural conditions in further studies on attempted culture of *M. lepraemurium in vitro.*

Although structures similar to the larger bodies have been demonstrated in several species of Mycobacterium, e.g., *M. avium* ([1, 12]), *M. johnei* ([26]), *M. leprae* ([9]), *M. lepraemurium* ([4, 10, 19]), *M. phlei* ([29]), *M. smegmatis* ([7]), *M. tuberculosis* ([14, 16]), BCG ([17, 21]), and unidentified Mycobacterium species ([10]), no real indication of their function or significance has been given. Rees *et al.* ([19]) described cells of *M. lepraemurium* markedly exhibiting these structures as segmented forms and found that these changed to degenerate forms upon continued incubation and were not viable. We found that upon careful examination of organisms from lepromas the large inclusions could be observed in many of the cells (both in negatively-stained preparations and in sections) and in varying degree. Some were small, some barely visible, while others were very pronounced and filled the whole or most of the cytoplasm ([1, 14, 17, 19]). Imaeda and Ogura ([10]) showed good sections of these structures but since their paper was not dealing with these structures they made no reference to them. Chapman *et al.* ([4]) who found the large bodies in *M. lepraemurium,* stated that the significance of these low density inclusions is unknown. Some of the inclusions designated as polyphosphate bodies by Imaeda and Convit ([9]) appear similar to our large bodies rather than to typical more electron-dense polyphosphate deposits. Kölbel ([14]) interpreted these structures as vacuoles which are surrounded by a thin membrane and produced by the phosphate granules. Merckx *et al.* ([16]), however, described the structures as spherules and found, in sectioned mouse lung tissue infected with *M. tuberculosis,* that the spherules could be released by rupture of the bacilli and that some may be embedded in lysosomes. Smith ([26]) found that in *M. johnei* the large electron translucent areas, which on occasion almost completely filled the available cytoplasm, were the most predominant features of the organisms in culture. He stated that these structures were most probably lipid and may be analogous to the oil red O-staining inclusions described in *M. smegmatis* by Gale and McLean ([7]). Gale and McLean ([7]) found that these inclusions occupied a greater volume in ethambutol-treated cells of *M. smegmatis.*

In whole cells examined electron-microscopically the large inclusions appear as definite structures with sharply defined edges. Brieger and Glauert ([1]) described them as round bodies of uniform density, each enclosed in a dense outer membrane. Also Gale and McLean ([7]) described them as appearing limited by a single-layered membrane. However, in sections (Figs. 5, 6, 7, 8) the edges do not appear well-defined and certainly do not appear as if they are bordered by a membrane. Their solid nature is demonstrated by the fact

that in degenerate cells the large bodies are frequently still present but the other cell contents seem to be reduced and the cell membrane outlines the inclusions, giving the appearance of a stocking stuffed with oranges. If they were liquid-filled vacuoles they would presumably collapse during drying of the specimens for electron microscopy or would burst and the liquid vaporize in the high vacuum of the electron microscope column. Because they are not membrane bound and because of their granular internal structure, and also because they maintain their shape in dried preparations, they most probably are not vacuoles. That they are spores was suggested by Brieger and Glauert ([1]) but this is unlikely because of their lack of a wall, membrane and any internal structure. They are quite different from nuclear material, which has a distinctly coarser structure in our preparations (even though uranyl acetate was used in the fixation procedure) and which is not so organized into spherical or near-spherical shapes as are the large bodies. They also bear no resemblance to mesosomes since they have no membranous components and are homogeneous.

Intracytoplasmic membranes have been demonstrated in several species of Mycobacterium, e.g., *M. avium* ([24, 25]), *M. avium* (Jucho strain) ([13, 28]), *M. johnei* ([26]), *M. leprae* ([3, 9, 10]), *M. lepraemurium* ([4, 10]), *M. phlei* ([5]), *M. smegmatis* ([7, 20]), *M. tuberculosis* v. *hominis* ([25]), BCG ([20]), and unidentified Mycobacterium species ([10]). Distinct membranous structures, now called mesosomes, were found in many organisms, including *M. lepraemurium* ([4, 10]). We have only one photograph (Fig. 10) illustrating a mesosome although extensive internal membranes were found in many organisms, and in some photographs (e.g., Figs. 5 & 11) these were shown to be continuous with the cell membrane ([3, 9, 10, 13, 20, 26]). Often the membranous structures of *M. lepraemurium* demonstrated in this investigation resemble those produced in *M. smegmatis* by bacitracin treatment ([20]) which causes derangement of plasma membranes and mesosomes. The organism in Figure 5 appears as though there is some fault in the normal mechanism of cell division and an excessive amount of membrane has been produced, instead of the normal amount, which would constrict the organism into two daughter cells.

SUMMARY

Small electron-dense particles (mean diameter *ca.* 30 mμ, range *ca.* 10 to 60 mμ) were found in cells of *Mycobacterium lepraemurium* isolated from mouse lepromas. They occurred most often in clusters and are probably granules containing metaphosphates. The smallness of these inclusions may be related to the metabolic inefficiency of *M. lepraemurium*.

The organisms also frequently contained larger bodies (mean diameter *ca.* 175 mμ, range about 120 to 300 mμ) which were not so electron-dense as the smaller inclusions and which in sections were seen to be finely granular, homogeneous masses of material not bordered by membranes. These are most probably deposits of a storage material such as lipid, but their exact nature awaits further investigation. The two types of inclusion were also seen in cells of *M. leprae*.

Elaborate membrane systems were often found in the cytoplasm and these were shown in some cases to be invaginations of the cell membranes, although only one typical mesosome was found in the specimens examined.

RESUMEN

Se encontraron partículas pequeñas (diámetro medio ca, 30 μ, rango ca 10 a 60 μ), densas a los electrones, en las células de *Mycobacterium lepraemurium* aisladas de lepromas de ratón. Se presentaban con mayor frecuencia en forma de acúmulos y son probablemente gránulos que contienen metafosfátos. Lo pequeño de estas inclusiones puede estar relacionado con la ineficiencia metabólica del *M. lepraemurium*.

Los microorganismos contenían también frecuentemente cuerpos mayores (diámetro medio ca. 175 μ, rango alrededor de 120 a 300 μ) que no eran tan densos a los electrones como las inclusiones más pequeñas y que se veían en los cortes como masas de material finamente granulado, homogeneo, no rodeadas de membranas. Estos son muy probablemente depósitos de material de reserva tal como

lípido, pero se necesitan estudios ulteriores para determinar su naturaleza exacta. Los dos tipos de inclusión se vieron también en células de *M. leprae*.

Con frecuencia se encontraron en el citoplasma complicados sistemas de membranas que en algunos casos se demostró eran invaginaciones de las membranes celulares, aunque se encontró sólo un mesosoma típico en las muestras examinadas.

RÉSUMÉ

Dans des cellules bactériennes de *Mycobacterium lepraemurium*, isolées de lépromes de souris, on a observé de petites particules denses aux électrons (diamètre moyen approximativement 30 μ avec une variation de 10 à 60 μ). Ces particules se présentent plus souvent sous forme d'amas, et représentent probablement des granules contenant des méta phosphates. La petite dimension de ces inclusions peut être mise en relation avec l'inefficacité métabolique de *M. lepraemurium*.

Les organismes étudiés contenaient fréquemment aussi des corpuscles de plus grande dimension (diamètre moyen approximativement 175 μ, avec une variation de 120 à 300 μ) qui n'étaient pas aussi denses aux électrons que les plus petites inclusions; en coupes, ces corpuscles apparaissaient comme des masses homogènes, finement granulaires, de matériel non délimité par des membranes. Ils consistent probablement en dépôt de matériaux de réserve, tel que des lipides, mais leur nature exacte devrait faire l'objet de verifications complémentaires. Les deux types d'inclusions ont également été relevés dans des cellules de *M. leprae*.

Des systèmes élaborés ont souvent été trouvés dans le cytoplasme. On a trouvé que dans certains cas, il s'agissait d'invaginations des membranes cellulaires, quoiqu'un seul mésosome typique ait été trouvé dans les échantillons examinés.

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