AN ELECTRON MICROSCOPE STUDY OF THE DISPOSITION AND FINE STRUCTURE OF *MYCOBACTERIUM LEPRAEMURIUM* IN MOUSE SPLEEN[1]

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The techniques of ultrathin sectioning and electron microscopy have been applied to cultures or suspensions of mycobacteria (Brieger and Glauert, 1956; Toda *et al.*, 1957; Shinohara *et al.*, 1957; Shinohara *et al.*, 1958). The provocative nature of the resultant observations was sufficient in itself to suggest the desirability of studying these organisms as they occur in tissues. This project seemed all the more worthwhile when it was realized that, although a wide variety of microorganisms have been examined using these techniques, only the work of Goodman and Moore (1956), Goodman *et al.* (1956), Meyer and Frank (1957), and Brieger and Glauert (1956) has considered, via this high resolution approach, the relationships between the host cell and the microorganism, and the fine structure of the microorganism under this more pertinent condition of growth in an infected cell.

MATERIALS AND METHODS

Pieces of spleen, approximately 1 mm³ in volume were removed from a mouse which had been infected with *Mycobacterium lepraemurium*, the causative agent of murine leprosy. These tissues were placed into a fixative which consisted of 2.5 ml of a buffer (9.714 g of sodium acetate·3H₂O and 14.714 g of sodium veronal, made up to 500 ml of solution), 1 ml of 8.5 per cent NaCl, 3 drops of 0.11 M CaCl₂, 2.25 ml of distilled water, 0.5 ml of 0.1 N HCl, and 6.25 ml of 2 per cent OsO₄. The pH of this fluid was 8.5. The pieces of spleen were allowed to remain in this fixative for 6 days at room temperature. This seemingly long period of fixation is necessitated by the impermeability of the mycobacteria. Preservation of fine structure was superior to that

obtained on fixation for 7 hr. Following fixation' the pieces were washed in a fluid consisting of the fixing solution minus the OsO₄, dehydrated by passage through a graded ethyl alcohol series, and were finally embedded in no. 1 gelatin capsules in a mixture of three parts normal butyl methacrylate to two parts ethyl methacrylate polymerized at 70 C in the presence of 1.5 per cent luperco CDB. Ultrathin sections were cut with a Porter-Blum ultramicrotome using glass knives prepared in the laboratory essentially as described by Latta and Hartmann (1950). The sections were floated off the knife edge on to a surface of 40 per cent acetone in distilled water. They were picked from this surface on 200 mesh copper screens on which a thin collodion film had been mounted. The sections were examined in an RCA EMU-2D electron microscope which had been fitted with a 0.015 inch externally centerable (Canalco) condenser aperture and a 50 μ aperture in the standard objective pole piece.

RESULTS AND DISCUSSION

Figure 1 represents a typical field from a section through mouse spleen infected with *M. lepraemurium*. It is readily apparent that the 6-day fixation has exceeded the optima for preservation of cytoplasmic structures in the infected tissue cells. It is also obvious that the bacilli are reasonably well fixed as judged by the absence of angular lacunae (Porter and Kallman, 1953) from the bacterial cytoplasm and by the rarity of microorganisms with ruptured or discontinuous walls. Very little evidence of shrinkage exists. As one would expect, not every sectioned bacterium exhibits all of the structural characteristics of an intact cell. Figure 1 does illustrate, however, most of these characteristics. These may be listed at this time and subsequently discussed at greater length. Capsule enclosing
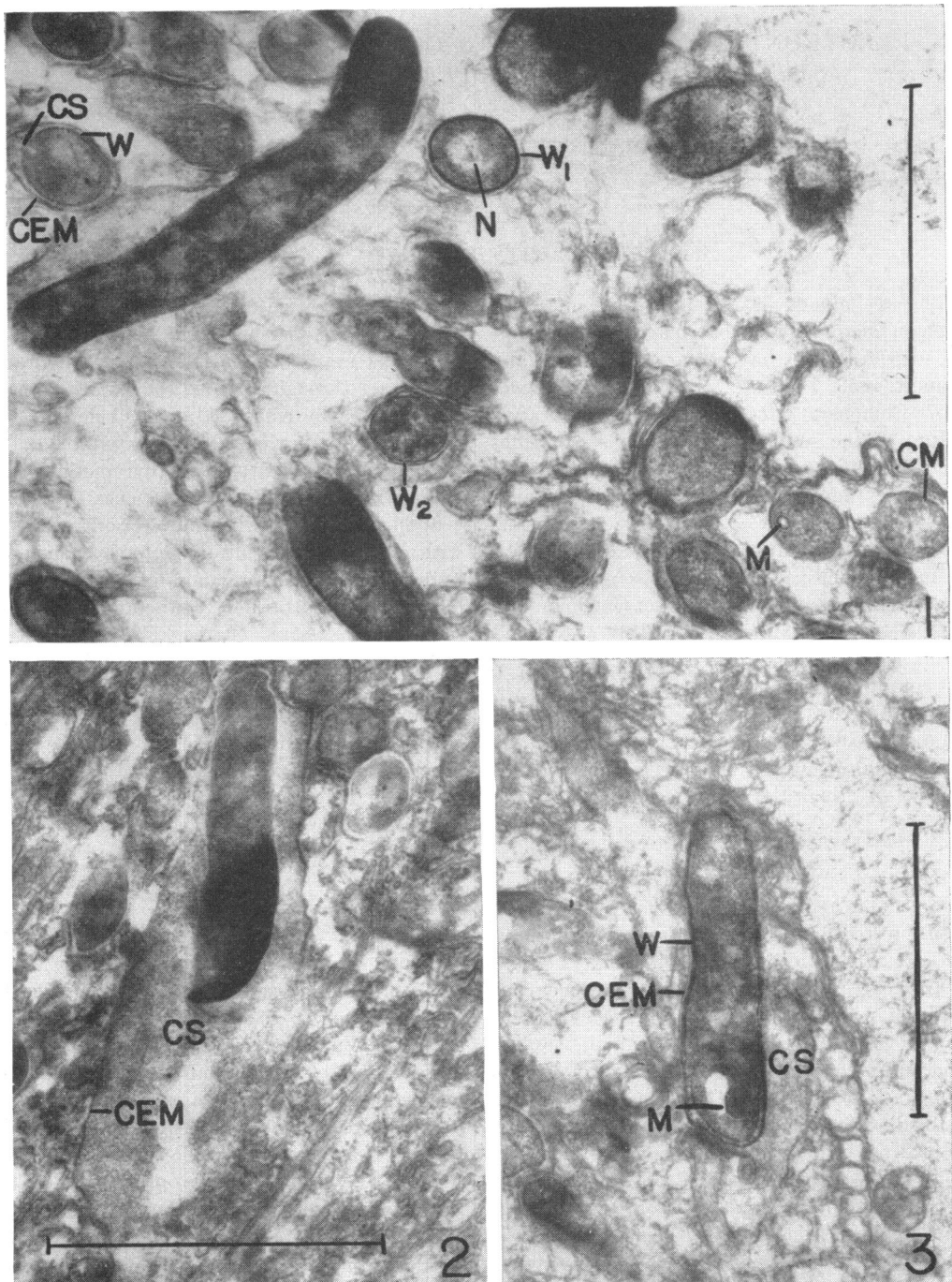
Figures 1, 2, and 3. Electron micrographs of ultrathin sections through mouse spleen infected with *Mycobacterium lepraemurium*. In each figure the magnification mark equals one micron. Figures 2 and 3 are examples of bacterial cells with large capsule spaces. *CEM*, capsule enclosing membrane; *CS*, capsule space; *W*, cell wall; *CM*, cytoplasmic membrane; *M*, mitochondrial equivalent; *N*, nuclear apparatus.

membrane (*CEM*) is the term applied to a membrane which seems to set the bacterial cell and its possible capsular material off from the host cytoplasm. Capsule space (*CS*) refers to the space between the capsule enclosing membrane and the bacterial cell wall (*W*). The cytoplasmic membrane (*CM*) of the bacterial cells is quite difficult to observe. It is labeled only in those cells in which slight shrinkage permits it to be demonstrated most convincingly. *N* denotes the low density areas in the bacterial cytoplasm which have come to be regarded generally as representing the bacterial nuclear material (Chapman and Hillier, 1953). The most striking cytoplasmic inclusion, designated *M*, appears usually as a sac of spheres. It is thought to correspond to the structures considered to be mitochrondrial equivalents by other workers. Low density inclusions, differing in appearance from the nuclear areas, occasionally are seen and are labeled *LDI*.

The capsule enclosing membrane, separating the spleen cytoplasm from the bacterial cells, is almost always detectable. This membrane may be closely apposed to the bacterial cell wall (figure 1, *CEM*) and be difficult to detect, or it may be rather loosely disposed about the bacterium (figures 2 and 3, *CEM*) and be readily seen. It is at present impossible to state whether this membrane is formed by the tissue cells or by the bacilli. However, after following the coursing of many of these membranes through the sections and seeing them, in some instances, become rather remote from the bacterial cells, it may be more reasonable to assume the former mode of origin. This interpretation would be consistent with the fact that in no case could any evidence be seen for the formation of the membranes by the bacteria and by the fact that occasionally two or even three bacteria are found within one membrane.

It should be noted that no evidences were observed of a deleterious effect of the host cells on the microorganisms, such as that described by Goodman *et al.* (1956) for both virulent and nonvirulent bacteria. This finding is similar to that of Meyer and Frank (1957) who state that a complete balance apparently exists between the animal cell and the contained symbiotic bacteria.

The term "capsule enclosing membrane" was chosen because the capsule space usually seems to be occupied by a substance which is somewhat more finely granular than the cytoplasm of the host cells, and because capsular structures frequently are evident around stained bacilli in preparations coated with India ink or nigrosin. The capsule enclosing membrane delimits the "vacuoles" described in the cytoplasm by Brieger and Glauert (1956). It is hoped that further study will clarify the nature of the material which occupies the capsule space.

It should be noted that the microorganisms were never observed within the nuclei of host cells. Figure 4 shows two bacilli seemingly located within such a nucleus, *SN*. Close inspection reveals, however, that they actually are located in the host cytoplasm but in a nuclear membrane invagination and that each possesses a capsule enclosing membrane.

The bacterial cell wall in profile may be represented by two configurations: (a) a solid ring 120 to 150 A in thickness (figure 1, W_1); (b) two concentric rings, each 40 to 50 A thick, separated by a low density space of 40 to 50 A (figure 1, W_2). The configuration which is assumed by the wall may depend upon whether osmium is deposited homogeneously throughout this structure, or whether an excess of osmium has been accumulated at its inner and outer surfaces. Visualization of the two concentric rings is enhanced by a slight separation of the inner and outer wall surfaces. A similar wall appearance has been described in *Bacillus megaterium* by Piekarski and Giesbrecht (1956) and by Kellenberger and Ryter (1958) in *Escherichia coli*. No further substructure has been observed in these walls.

When the cell wall is closely appressed to the protoplast, the cytoplasmic membrane is rendered indistinguishable (figure 1, W_1). When a slight shrinkage space occurs between the protoplast and the cell wall, the cytoplasmic membrane is most easily observed (figure 1, *CM*). It appears to be about 30 A thick.

Low density areas, considered to represent nuclear material, were observed in many of the sectioned bacterial cells. Cells which illustrate this configuration (*N*) most convincingly appear in figures 1 and 4 to 7. The nuclear area in figure 5 is particularly interesting for it has assumed a dumbbell configuration reminiscent of that described by Chapman and Hillier (1953) in *Bacillus cereus* and includes several 40 to 60 A
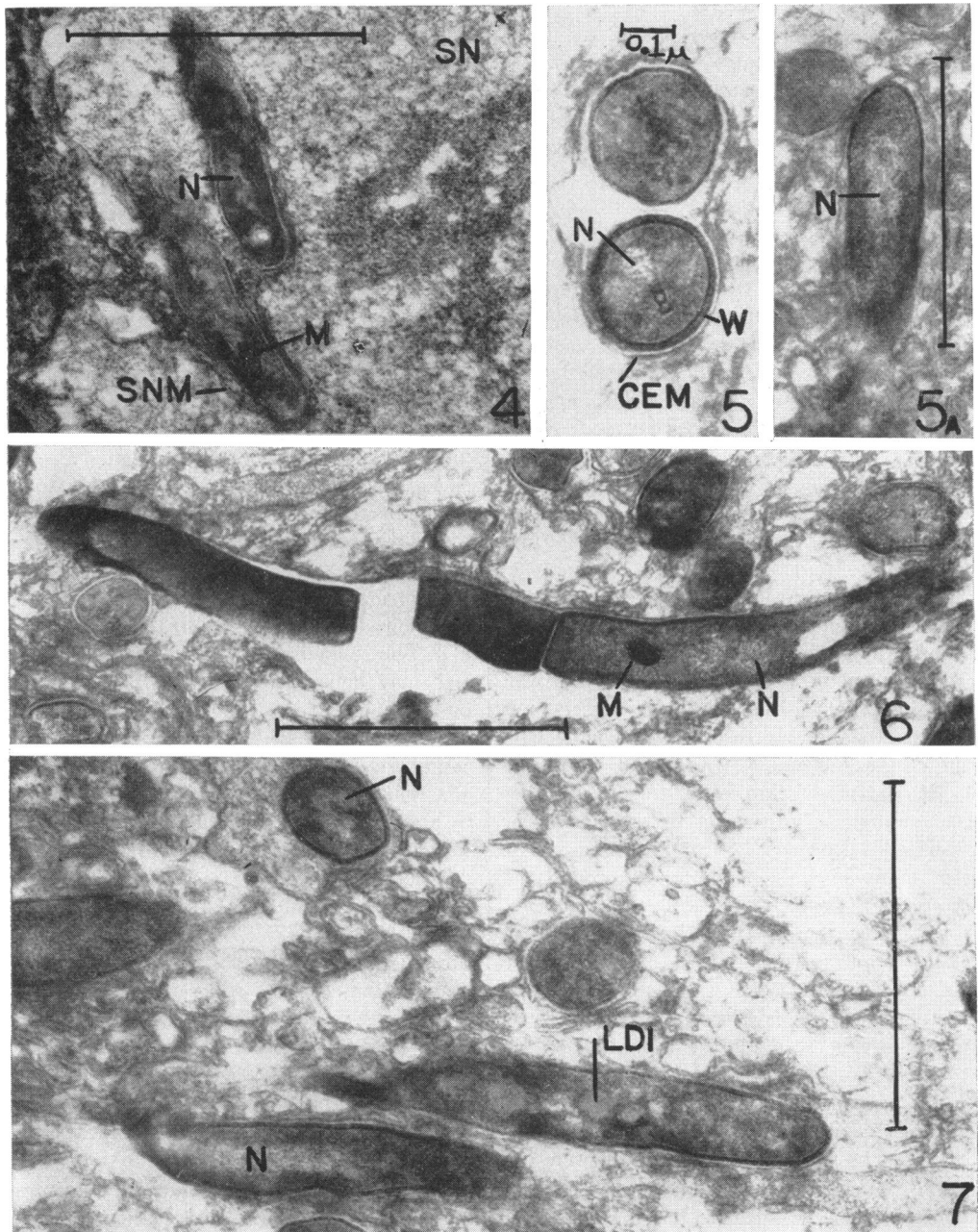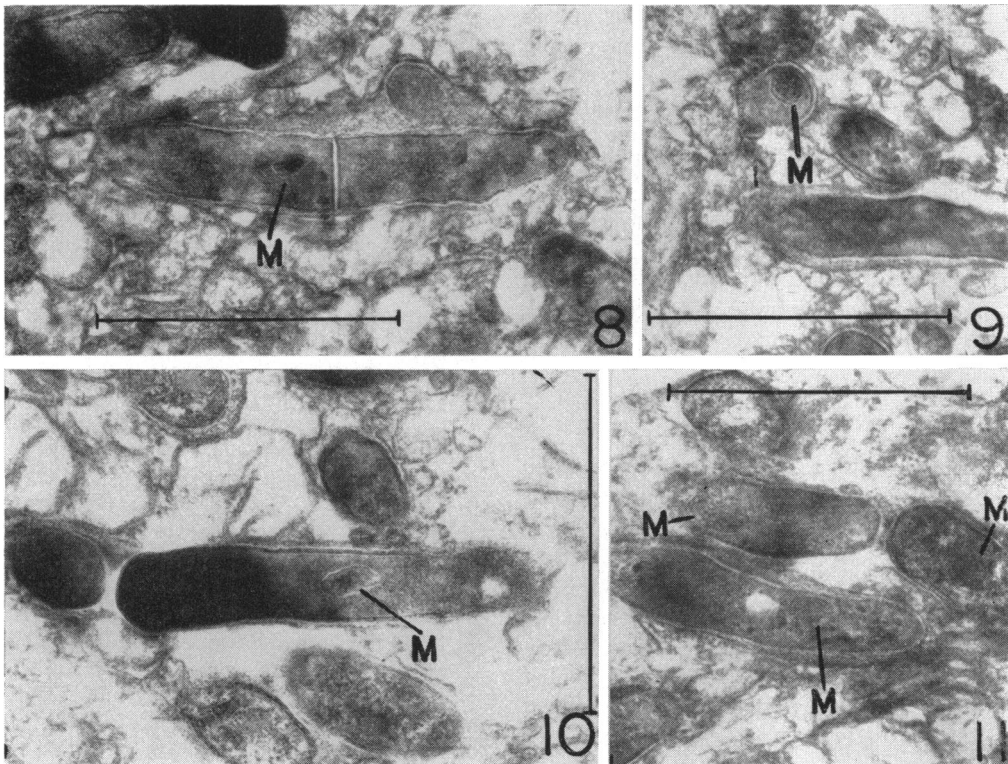
Figures 4 to 7. Electron micrographs of ultrathin sections through mouse spleen infected with *Mycobacterium lepraemurium.* The magnification marks equal one micron, unless otherwise indicated. Figure 4 shows two bacterial cells lodged within a nuclear membrane invagination. Figure 5, at high magnification, shows the nuclear area of one cell and the way in which the cell wall varies in appearance. Here, two cells are included within one capsule enclosing membrane. Figure 5A illustrates the dumbbell configuration of the nuclear material. Figure 6 shows three cells which have derived from a single cell by cellular division. Note the dense body, *M*, assumed to be a mitochondrial equivalent. Figure 7 illustrates the appearance of the low density inclusions. *SN*, nucleus of spleen cell; *SNM*, nuclear membrane of spleen cell; *LDI*, low density inclusion.

thick threads of dense material similar to those described by Kellenberger and Ryter (1955) in *E. coli*. The nuclear area, *N*, in figure 1 includes several dense granules which may represent cross sections through these threads. It is interesting to note that Caro *et al.* (1958), employing the methods of radioautography, have found that, when a thymine-requiring strain of *E. coli* is grown in a medium containing tritium-labeled thymidine, the radioactivity is found in the low density areas. Thus, observations from yet another discipline corroborate the interpretation that it is these areas which represent the bacterial nuclear material.

The dense, membrane-limited inclusions, *M*, in figures 1, 3, 4, 6, and 8 to 11 may exceed 1200 A in diameter. They have also been illustrated by Toda *et al.* (1957) and by Shinohara *et al.* (1958). The earlier authors described these structures as revealing "no internal structure in ultra-thin section other than the artifactual 'honey-comb' or 'chrysanthemum' appearance caused by partial volatilization under electron bombardment." The electron micrographs of the latter authors reveal more strikingly the nature of these inclusions, as do the figures presented in this paper. Careful examination of the inclusions leads one to the impression that their most general appearance is that of a body limited by a 40 to 60 A membrane and containing a number (perhaps 25 to 50) of nearly spherical granules, each approximately 200 A in diameter. Since exposure of the sections to relatively low electron beam intensity reveals the honeycomb appearance, it seems that this configuration is not caused by electron bombardment. It is interesting to note that figures 15 and 16 of Mudd *et al.* (1956) reveal the occurrence of dense granules, of the same order of magnitude as the above spherical granules, within larger low density bodies. It seems likely that these two configurations represent identical structures. It should

Figures 8 to 11. Electron micrographs of ultrathin sections through mouse spleen infected with *Mycobacterium lepraemurium*. The magnification marks equal one micron. Figure 8 shows a mitochondrial equivalent, appearing as a group of spheres, in a recently divided cell. The recently split transverse cell walls are readily seen. Figures 9, 10, and 11 illustrate the appearance of mitochondrial equivalents.

be noted that, although the small particles change their appearance on intense electron bombardment, there is no problem in identifying them under the two types of observational conditions, i. e., high and low beam intensities. On the basis of the work by Mudd and his coworkers (summarized and cited in Mudd *et al.*, 1956) and by Shinohara *et al.* (1958), it seems reasonable to assume that the cytoplasmic inclusions in mycobacteria, variously called mitochondria, mitochondrial equivalents, and sites of oxidation-reduction, do correspond to the membrane-limited, spherical, granule-containing bodies described herein. It seems reasonable to call these bodies bacterial mitochondria just as the mitochondria of protozoa, with their tubular interna, are called mitochondria. The attribute of possessing cristae mitochondriales need not be a requisite for classification as a mitochondrion.

The low density inclusion labeled *LDI* in figure 7 is presumed to be different in nature from the low density areas labeled *N* in this and in other figures. The areas labeled *N* appear more granular and occasionally exhibit threads of material whereas the area labeled *LDI* appears to be almost agranular or at least much more finely granular. The significance of this type of inclusion is unknown.

Figures 6 and 8 illustrate bacilli which have recently completed cellular division. The completed cross walls are particularly striking in figure 8. No sections were obtained through organisms which were in the actual process of cellular division.

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SUMMARY

Study of ultrathin sections of methacrylate embedded mouse spleen infected with *Mycobacterium lepraemurium* has revealed a number of interesting facts. The bacteria are separated from the host cytoplasm by a capsule enclosing membrane which seems to be derived from the host cytoplasm. The space between this membrane and the bacterial cell wall is occupied by a finely granular material which may represent a bacterial product or capsule. Those bacilli which

appeared to be within the nuclei of host cells were in fact separated from the nuclear contents by invaginated nuclear membranes.

The bacterial cell walls are about 150 A thick and may be closely appressed to the cytoplasmic membrane or separated from it by a narrow shrinkage space. When the latter exists, a 30 A thick cytoplasmic membrane is clearly visible. No substructure was observed in either cell wall or cytoplasmic membrane. However, the cell wall occasionally appears double due to an excess accumulation of dense material (presumably osmium) at its two surfaces.

Low density areas, assumed to be the nuclear material, have been observed. They sometimes exhibit granular threads of denser material.

Membrane-limited inclusions, apparently containing numerous spherical particles, are seen in many sectioned bacilli. These presumably are the equivalents of mitochondria within such cells.

Occasionally, a low density inclusion, different in appearance from the nuclear areas, was observed.

Several bacteria have been observed which had just completed cellular division when they were fixed.

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