ELECTRON MICROSCOPE STUDY OF *MYCOBACTERIUM LEPRAE* AND ITS ENVIRONMENT IN A VESICULAR LEPROUS LESION

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ABSTRACT

IMAEDA, TAMOTSU (Instituto Venezolano de Investigaciones Cientificas, Caracas, Venezuela) AND JACINTO CONVIT. Electron microscope study of *Mycobacterium leprae* and its environment in a vesicular leprous lesion. J. Bacteriol. **83**:43–52. 1962.—Biopsied specimens of a borderline leprosy lesion were observed with the electron microscope. In this lesion, the majority of *Mycobacterium leprae* were laden with cytoplasmic components. The bacilli were separated from the cytoplasm of host cells by an enclosing membrane, thus differing from the environment of well-developed lepra cells in lepromatous lesions.

The cell wall is composed of a moderately dense layer. A diffuse layer is discernible outside the cell wall, separated from it by a low density space. It is suggested that the cell wall is further coated by a low density layer, although the nature of the outermost diffuse layer has not yet been determined.

The plasma membrane consists of a double layer, i.e., dense inner and outer layers separated by a low density space. The outer layer is closely adjacent to the cell wall. In the region where the outer layer of the plasma membrane enters the cytoplasm and is transformed into a complex membranous structure, the inner layer encloses this membranous configuration. Together they form the intracytoplasmic membrane system.

In the bacterial cytoplasm, moderately dense, presumably polyphosphate bodies are apparent. As neither these bodies nor the intracytoplasmic membrane system are visible in the degenerating bacilli, it seems probable that these two components represent indicators of the state of bacillary activity.

Submicroscopic structures of *Mycobacterium leprae* have been studied with the use of both ultrathin sections of leprous lesions and bacilli separated from lesions (Bishop, Suhrland, and Carpenter, 1948; Malfatti, 1951; Yamamoto et al., 1958*a*; Chatterjee, Das Gupta, and De, 1959; De Souza-Araujo, 1960; McFadzean and Valentine, 1960). In none of these cases, however, was the cytoplasmic structure of the bacilli clearly defined.

Recently, Brieger, Glauert, and Allen, (1959) observed an intracytoplasmic membrane in this bacillus, using organ culture of lepromas.

Fortunately, we encountered a biopsied tissue which contained many bacilli and disclosed their fine structure. The present study was undertaken to elucidate the submicroscopic structure of *M. leprae* and its bacillary environment, which have never been observed before in biopsied specimens.

MATERIALS AND METHODS

The material employed in this study was a biopsied specimen from a case of borderline leprosy which had been diagnosed both clinically and histopathologically. This lesion showed reddish infiltration and a small blister.

The specimen was fixed with 1% osmium tetroxide, buffered with 2,4,6-collidine (0.2 M) at pH 7.35, for 6 hr (Hama, 1959). Tissue chips were dehydrated with acetone and embedded in a mixture of *n*-butyl (80%) and methyl methacrylate (20%). Ultrathin sections were obtained by means of an ultramicrotome (Leitz Ultra-Mikotom, Fernández-Morán, Germany) equipped with a diamond knife. After sectioning, some of the ultrathin sections were treated for 15 min with 2% uranyl acetate solution (modification of the method of Watson, 1958). The electron microscope used was a Siemens Elmiskop I at 60 kv.

To demonstrate *M. leprae* in a leproma, one of the nodules of a lepromatous patient was examined in the same manner as mentioned above.

RESULTS AND DISCUSSION

Bacillary environment in the host cell. Electron micrographs of this lesion show many bacilli distributed in the cytoplasm of the histiocytic cells

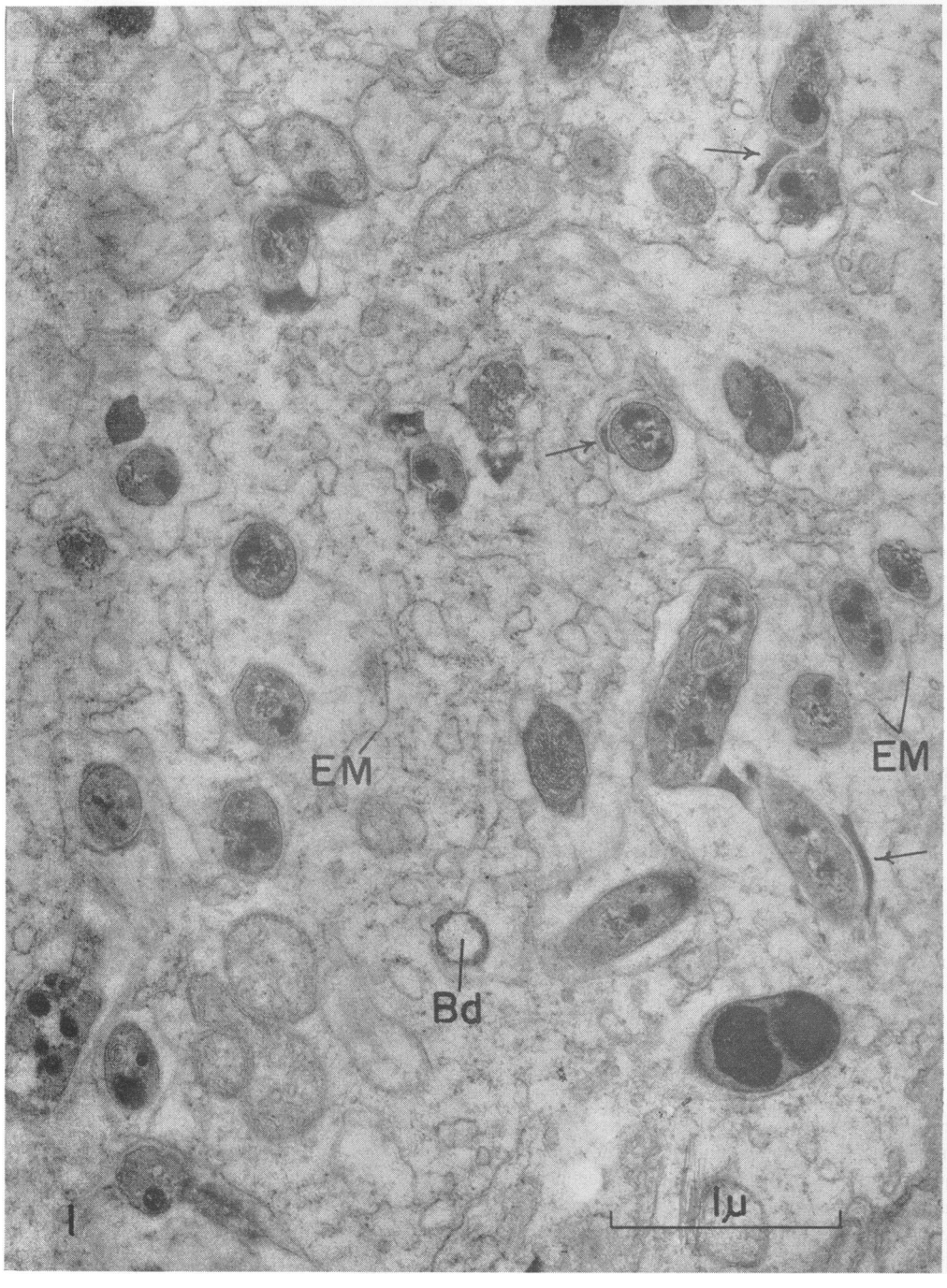
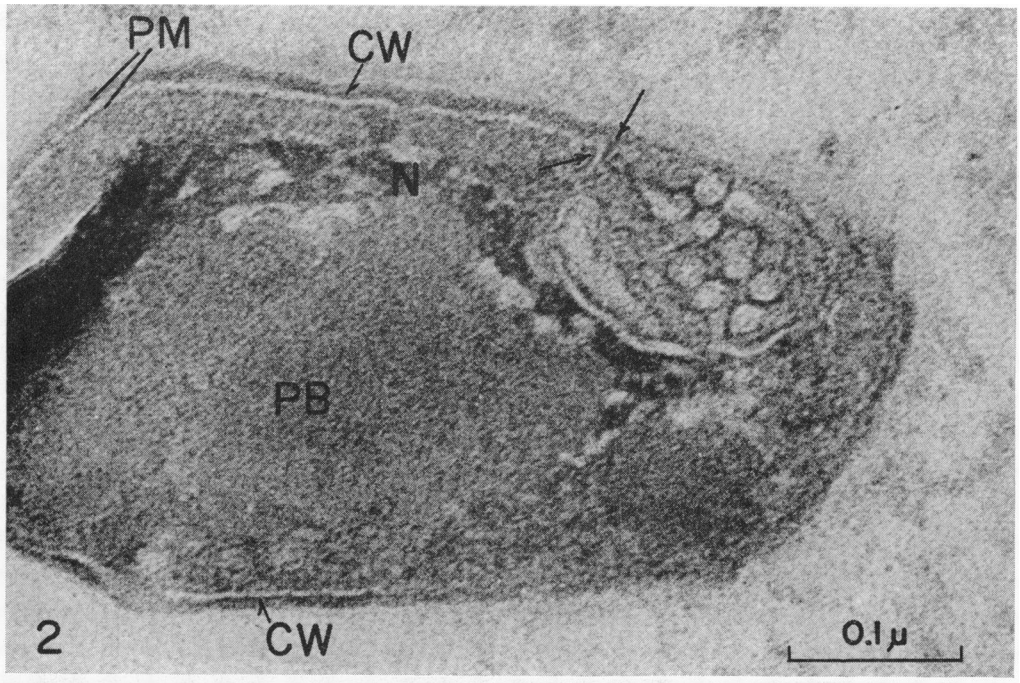


FIG. 1. *Mycobacterium leprae abundantly distributed in the cytoplasm of the host cell. These bacilli are separated from the cytoplasm by the enclosing membrane (EM). A homogeneously dense substance, supposedly the remnant of opaque droplets, attaches to the bacilli (arrow). In the lower part of this picture, a bacillus (Bd) shows the cytoplasmic condensation.*



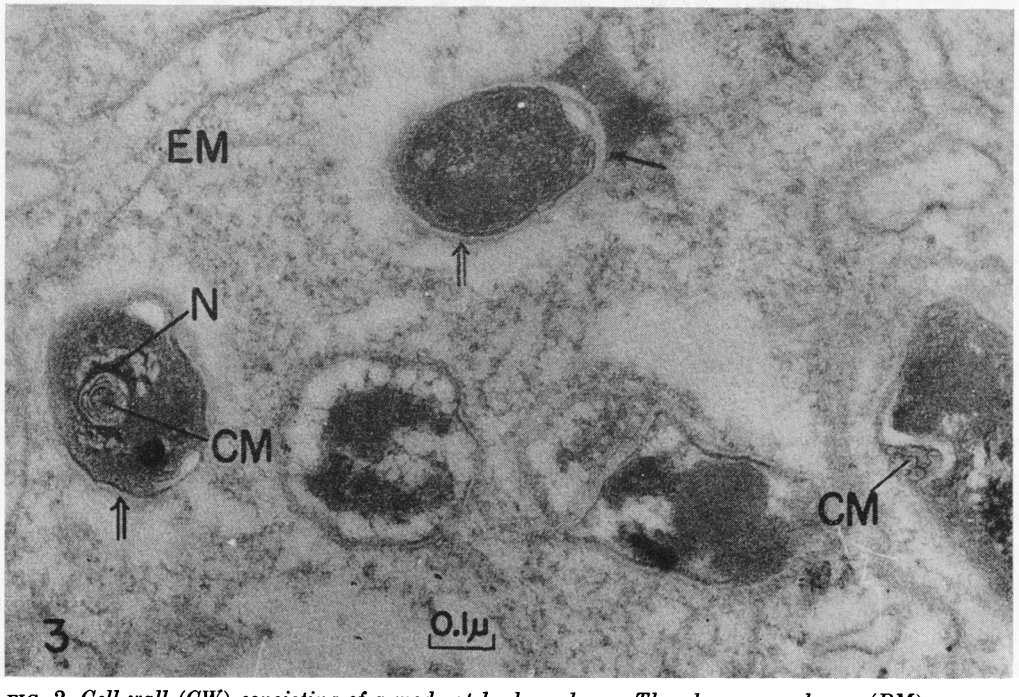


FIG. 2. *Cell wall (CW) consisting of a moderately dense layer. The plasma membrane (PM) appears to be composed of a double layer separated by a low density space. The inner layer here seems to recede with the bacterial cytoplasm. The outer layer of the plasma membrane adheres closely to the cell wall. In a region indicated by arrows, the plasma membrane enters the bacterial cytoplasm and is transformed into an intracytoplasmic membrane system, generally limited by the inner layer of the plasma membrane. Nuclear apparatus (N) is visible in a low density area. PB: polyphosphate body.*

FIG. 3. *Slightly dense, diffuse layer (double arrow) is separated from the cell wall by a low density layer. The latter is also apparent between the cell wall and the clinging dense substance (arrow). CM: intracytoplasmic membrane system.*

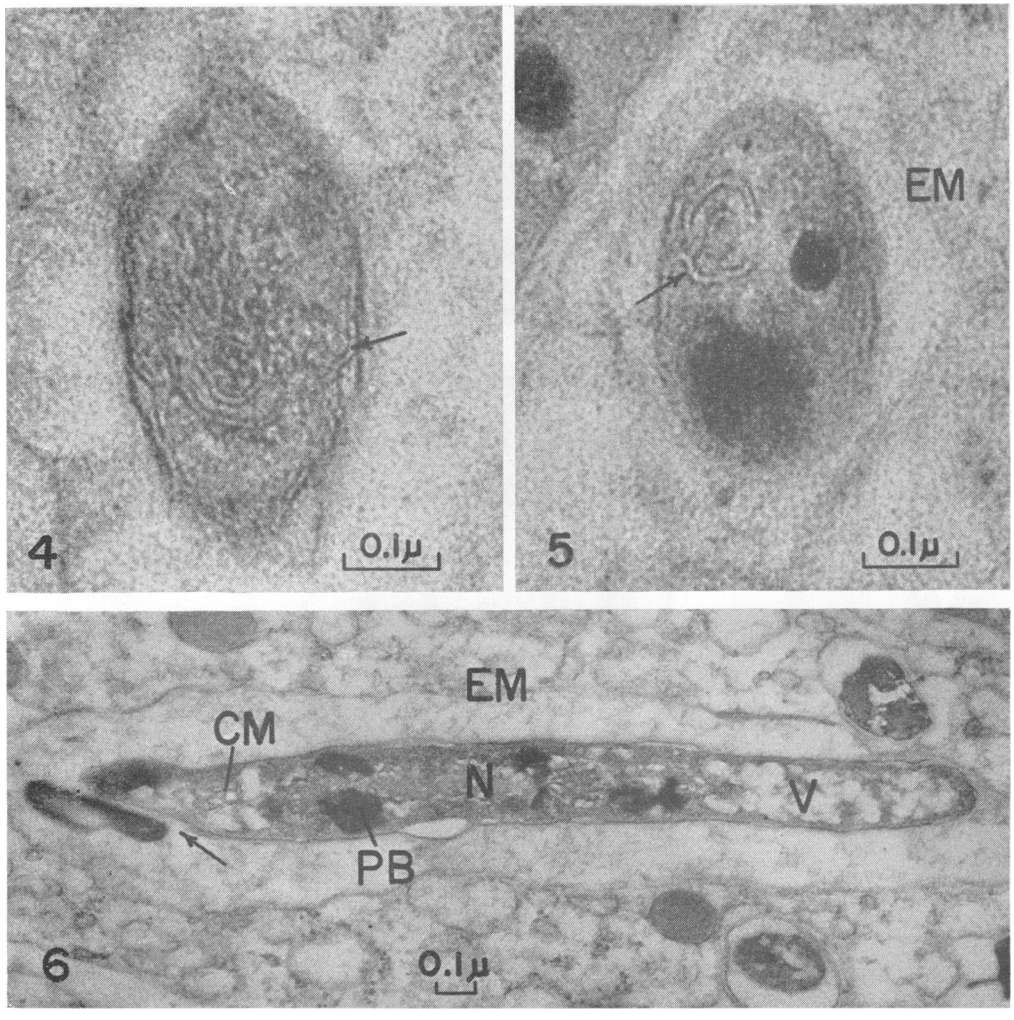


FIG. 4 and 5. *Larger magnifications of two bacilli in Fig. 1. These pictures exhibit the relationship between the intracytoplasmic membrane system and the plasma membrane (arrow).*

FIG. 6. *A long bacillus containing vacuoles (V) at one end of the cytoplasm. Polyphosphate bodies show an irregular shape near the nuclear region. The remnant of the opaque droplet does not attach directly to the cell wall, but is separated by a low density space (arrow).*

(Fig. 1). The bacilli are not surrounded by a moderately dense substance, which was called "opaque droplet" by Yamamoto et al. (1958*a*, *b*), but are enveloped by a membrane separating them from the cytoplasm of the host cells. This enclosing membrane, clearly distinguishable from the bacterial cell wall and sometimes including several bacilli (Fig. 1 and 3), may be the remnant of the host cell membrane taken into the cytoplasm when the bacilli were phagocytized, as suggested by Chapman, Hanks and Wallace (1959) in their study of *M. lepraemurium*. The space inside the enclosing membrane is usually electron transparent, but occasionally contains fine granules.

It should be noted that the enclosing membrane observed in this lesion does not frequently appear in the case of lepra cells. In lepromas, a host-cell reaction to bacillary reproduction occurs inactively and, in consequence, the bacilli proliferate rapidly, forming glomerations termed "globi". Furthermore, a single bacillus, or a group of bacilli, is surrounded by an opaque droplet and foamy structure (Fig. 9), probably of

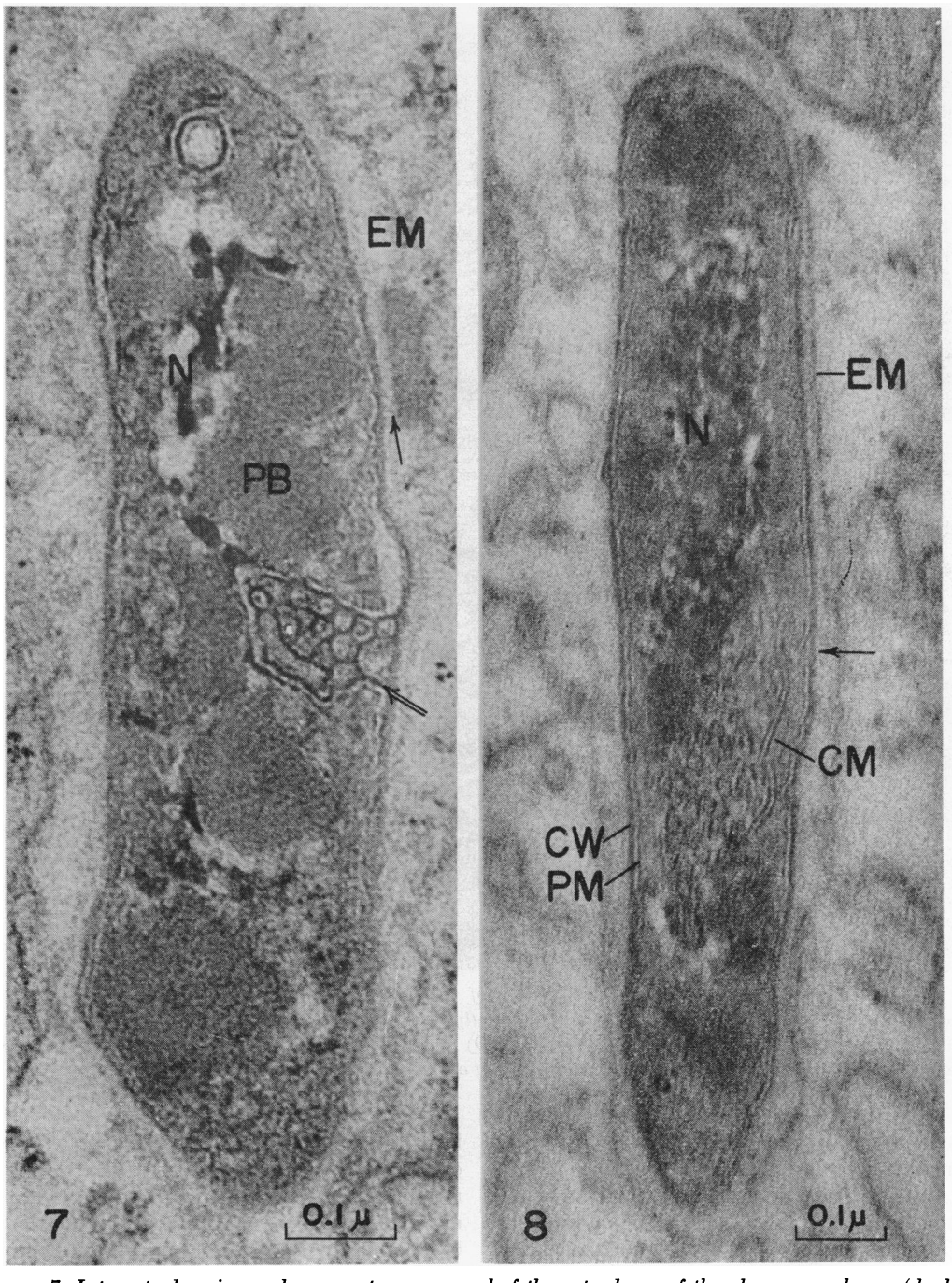


FIG. 7. *Intracytoplasmic membrane system composed of the outer layer of the plasma membrane (double arrow) and the enclosing inner layer of the plasma membrane. A low density space (arrow) is visible between the cell wall and the remnant of the opaque droplet.*

FIG. 8. *Parallel arrangement of the intracytoplasmic membrane system (CM). In the region indicated by an arrow, both the inner and outer layers of the plasma membrane are taken into the cytoplasm.*

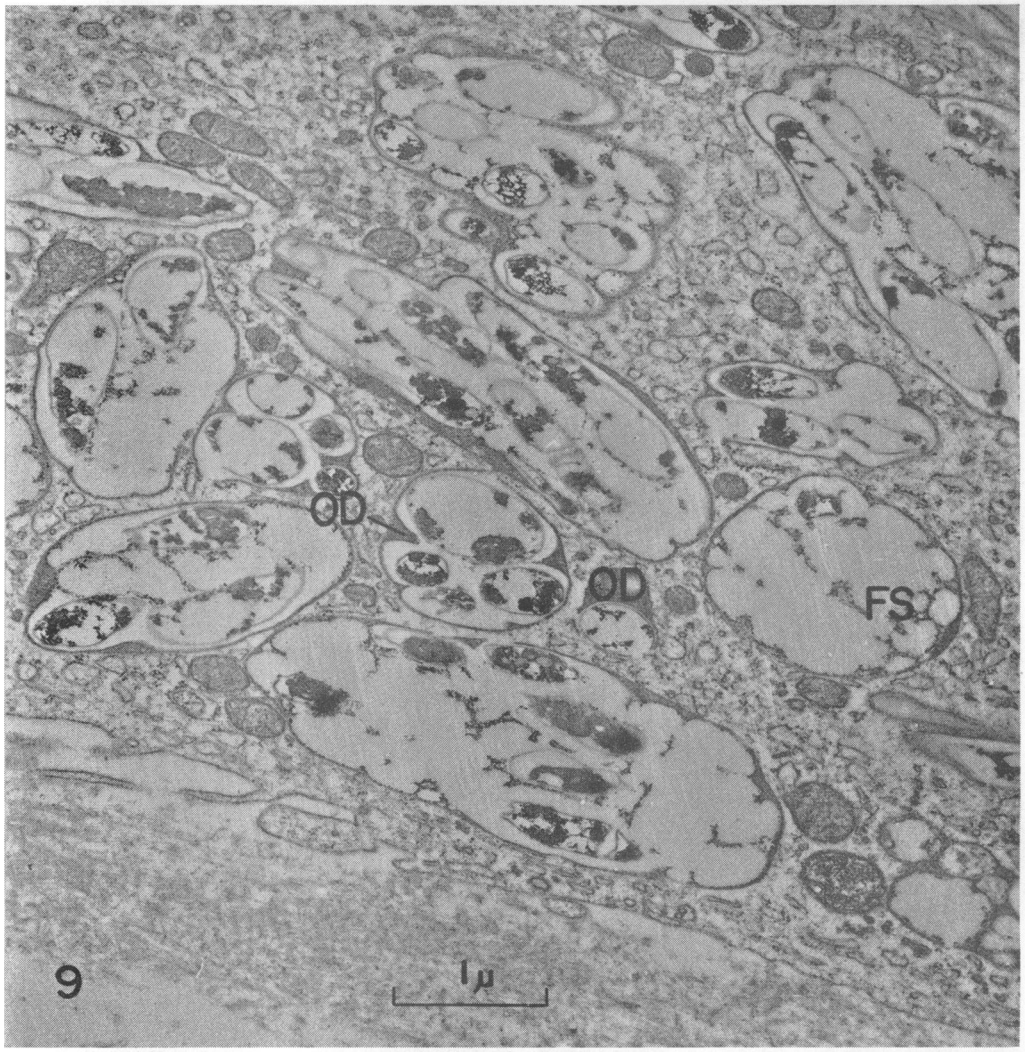


FIG. 9. *A lepra cell in a well-developed leproma of another lepromatous patient. The majority of the bacilli show cytoplasmic condensation, and consequently their fine structures are not clearly observed. These bacilli are partially enclosed by opaque droplets (OD) and foamy structures (FS). Note the difference between the bacillary environment of this lesion and that of the borderline lesion shown in other figures.*

lipid nature, as a result of the abnormal lipid metabolism of the cell in lepromatous leprosy (Imaeda, 1960).

An osmiophilic substance, thought to be the remnants of opaque droplets, was found clinging to the bacilli within one enclosing membrane (Fig. 1, 3, 6, and 7). From this evidence it is surmised that the cells containing the bacilli, wrapped in opaque droplets, may be broken down through a destructive process, and that the bacilli released to extracellular spaces may be phagocytized again by the histiocytic cells.

In other words, one may be able to observe the initial stage of cytoplasmic changes after phagocytosis. During a secondary stage these enclosing membranes may disappear and opaque droplets may take their place by wrapping around the bacilli. Of course, this process occurs only in borderline and lepromatous leprosy lesions. The bacillary environment in this lesion is quite different from that of any other well-developed leproma, although the process is the same.

Bacterial cell wall and plasma membrane. It has already been reported by Yamamoto et al.

(1958*a*) that the cell wall of *M. leprae* consists of three layers. In the studies of other mycobacteria, such as *M. avium*, *M. tuberculosis* var. *hominis* H37Rv and B.C.G., and *M. lepraemurium*, it has been shown that their cell walls are also composed of three layers (Fukushi, 1959; Chapman et al., 1959; Shinohara et al., 1959; Takeya, 1959).

In the present study, the cell wall, which appears to be 6 to 10 mμ wide, shows a moderately dense layer (Fig. 2). Adhering closely to the cell wall, a dense layer approximately 3 mμ thick is visible. This dense layer is believed to be the outer layer of the plasma membrane, as discussed in a later paragraph.

Outside the cell wall, a slightly dense, diffuse layer, separated from the cell wall by a low density space, is frequently discernible (Fig. 3 and 7). When moderately dense droplets attach to the bacilli, a low density space is always visible between the cell wall and the droplets (Fig. 1, 3, 6, and 7). Since these droplets are closely adjacent to the outermost diffuse layer of the cell wall, the space between the cell wall and the droplets may not be simply a shrinkage space. Therefore, this finding may be indicative of the fact that the cell wall is coated by a nonosmiophilic substance, supposedly of a waxy nature.

It is difficult to determine whether the slightly dense diffuse layer covering the outermost surface around the bacillus belongs to one of the components of the bacillary surface, or whether it is a host-cell product absorbed on the coating substance of the cell wall.

Many bacilli in the lepromas (Fig. 9) and also in the later stage of reactional tuberculoid lesions (Nishiura, 1960) show a cytoplasmic condensation which is believed to be a sign of bacillary degeneration. In the initial stage of the bacillary degeneration, the coating substance is still observed, although the bacilli already show the cytoplasmic condensation. However, this coating substance is no longer visible in the degenerated bacilli in either the old leproma or the later stage of reactional tuberculoid lesions. Based on these facts, it would seem that the coating substance around the bacterial cell wall may disappear in the last stage of bacillary degeneration.

The plasma membrane is composed of a double dense layer of about 30 A in thickness, separated by a low density space of about 30 A in width (Fig. 2, 4, 5, 7, and 8). The outer dense layer is in the most intimate contact with the cell wall, and consequently no space is apparent between them. The inner layer of the plasma membrane borders on the bacterial cytoplasm. In some regions, two layers of the plasma membrane enter the cytoplasm and are transformed into the membranous configuration (Fig. 2, 4, 5, and 7).

In studies of the *Mycobacterium* Jucho strain (Koike and Takeya, 1961) and *Bacillus medusa* (Fitz-James, 1960), the plasma membrane of these bacilli also appears to be a double layer and has similar dimensions to *M. leprae*, but it is clearly separated from the cell wall by a low density space. On the other hand, Van Iterson (1961) reported in her study of *B. subtilis* that the outer layer of the plasma membrane is adjacent to the cell. This being so, the submicroscopic structure of the plasma membrane of *M. leprae* resembles that of *B. subtilis* rather than those of the Jucho strain and *B. medusa*.

Nuclear apparatus. Electron microscopic studies of bacterial cells have established that the bacterial nucleus includes threads of dense material, 30 to 60 A in diameter, when especially refined fixation and embedding methods are employed (Ryter and Kellenberger, 1958; Takeya, 1959; Glauert, Brieger, and Allen, 1961; Van Iterson and Robinow, 1961). On the other hand, Chapman et al. (1959) failed to demonstrate these fine threads in the nucleus of *M. lepraemurium*.

Figures 2, 3, 6, 7, and 8 reveal an irregularly shaped, dense material in the transparent vacuoles. This is considered to represent the nuclear apparatus, which, however, does not correspond to its true visualization because of an artifact caused by the shrinkage of the nuclear material during preparation, as described by Takeya (1959). From this point of view, the preparation used in our study is inappropriate for a discussion of the fine structure of nuclear apparatus.

Polyphosphate body. Homogeneous, dense substances are seen in the bacterial cytoplasm. These substances appear to be round or oval, sometimes as irregular masses (Fig. 1, 2, 3, 6, and 7). They are not delimited by any membrane and apparently have no internal structures, although they are easily distinguishable from the granular matrix of the cytoplasm. There is often visible a granular appearance caused by electron bombardment.

As these substances never evidence any feature showing shrinkage, it is assumed that they are composed of osmiophilic matter which is not de-

formed through preparation for electron microscope examination, thus differing from the nuclear substance.

It is well known that *M. tuberculosis* also contains a dense substance, called "A-Body", which is easily vacuolated by electron bombardment. Takeya et al. (1954), Mudd, Takeya, and Henderson (1956), Mudd, Yoshida, and Koike (1958), and Takeya (1959) concluded that this body represents neither the nuclear apparatus nor the mitochondria, but rather a polyphosphate as an energy source of the bacilli.

The morphological appearance of the dense substance in *M. leprae* shows a close similarity to that of *M. tuberculosis*. It is therefore supposed that the dense substance in *M. leprae* also comprises a polyphosphate, although this has not yet been proved. Drews (1960) suggested in his study of *M. phlei* that the polyphosphate body might originate from irregularly shaped intermediate stages or from an agglomeration of the microgranules. However, the fact that irregularly shaped dense bodies generally appear near the nuclear region suggests that they may probably be related to the metabolism of the bacillary nuclei.

It should be emphasized that polyphosphate bodies never appear clearly in the degenerating bacilli contained in lepra cells (Fig. 9). In the lesion observed in this study, the bacilli were in an active condition, as discussed earlier. Based on this evidence, it is suggested that the presence of the polyphosphate body may be one of the indicators of the activity of *M. leprae* in host cells.

Intracytoplasmic membrane system. Complex, membrane-limited structures are found in the bacterial cytoplasm. They show various configurations, some beehivelike, others convoluting or lamellar, consisting of a dense layer approximately 30 A thick (Fig. 2, 3, 4, 5, 6, and 7). As seen in Fig. 2, 5, and 7, this membrane system shows a close connection with the outer layer of the plasma membrane.

In some regions, the enclosing membrane and the convoluted membrane are seen to be parallel, being separated by a low density space of 30 A in width (Fig. 8), and appear similar to the unit membrane described by Robertson (1959) in his studies on animal cells. The parallel membrane system observed by Brieger et al. (1959) in the cytoplasm of *M. leprae* may correspond to the particular arrangement of these two membranes.

From their studies of other bacilli, such as *B. medusa*, *B. megaterium*, and *Mycobacterium* Jucho, Fitz-James (1960), Giesbrecht (1960), and Koike and Takeya (1961) showed that the intracytoplasmic membrane system is derived mainly from a branching of the plasma membrane. On the other hand, Van Iterson (1961) observed that the membranous organelles of *B. subtilis* are in contact with the plasma membrane or with the cell wall. From our studies with methacrylate-embedded material, it would seem highly probable that the intracytoplasmic membrane system of *M. leprae* is composed of the outer layer of the

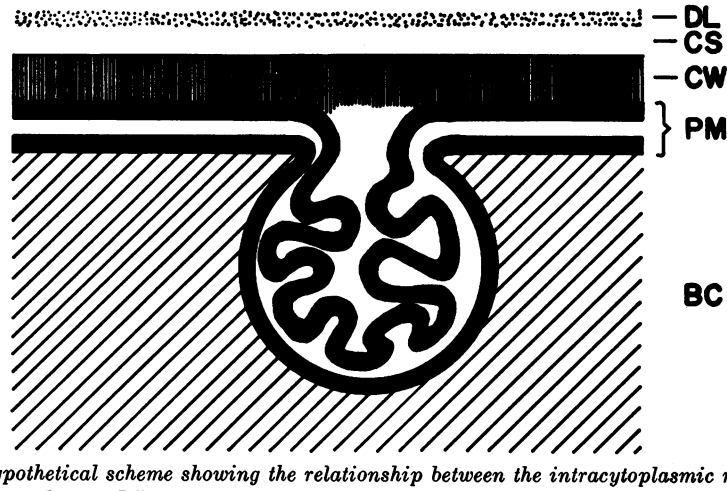


FIG. 10. *A hypothetical scheme showing the relationship between the intracytoplasmic membrane system and the plasma membrane. DL, diffuse layer outside the cell wall; CW, cell wall; PM, plasma membrane; BC, bacillary cytoplasm; and CS, coating substance.*

plasma membrane, which is surrounded by the inner layer (Fig. 10). Thus, the intracytoplasmic membrane system of *M. leprae* is quite similar to those of *B. subtilis*, *B. medusa*, *B. megaterium*, and the Jucho strain of *Mycobacterium*. However, further studies using other preparative methods will be necessary to clarify the relationship between the intracytoplasmic membrane system and the plasma membrane or cell wall.

The intracytoplasmic membrane system may represent one of the sites of energy-yielding enzyme systems in this bacillus, since it consists of the plasma membrane in which bacterial enzymes may be located, as postulated by Weibull and Bergstrom (1958) and Mitchell (1959). It is believed, therefore, that this structure may be one of the bacterial organelles related to metabolism, as observed in other mycobacteria and streptomyces (Shinohara, Fukushi, and Suzuki, 1957; Shinohara et al., 1958*a*, *b*, 1959; Takeya et al., 1958; Niklowitz, 1958; Toda, Takeya, and Koike, 1958; Takeya, 1959; Fukushi, 1959; Chapman et al., 1959; Zapf, 1959; Drews, 1960; Glauert and Hopwood, 1960; Koike and Takeya, 1961). Furthermore, this structure is not revealed in the degenerating bacilli showing cytoplasmic condensation. From this evidence, it may be concluded that such a membrane system appears only in active bacilli.

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