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The Interpretation of the Ultrastructure of Mycobacterial Cells in Transmission Electron Microscopy of Ultrathin Sections[1]

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The observation of ultrathin sections of bacterial cells by transmission electron microscopy is essential in many studies of bacterial physiology, pathology, taxonomy, host-parasite interactions, etc. The improvement in the techniques for ultrastructural observation, both in the preparation of the samples (better methods of fixation, better plastics for embedding, better ultramicrotomy) and in the microscopical observation (better resolution of the microscopes, image enhancement and filtration), helped considerably in the progressive elucidation of bacterial microanatomy. For example, in the initial electron-micrographs of sectioned bacteria no membranes were visible ([3]); now, we not only are able to visualize bacterial membranes quite clearly but also we are able to detect ultrastructural differences between the cytoplasmic membranes of Gram-positives and Gram-negatives ([17,25]) or between the membranes of *Mycobacterium leprae* and cultivable mycobacteria ([20,21,22]). It is important to realize, however, that as the techniques for the study of the fine structure of bacteria, or of any other biological entity, become more perfected, more care has to be taken in the preparation of the samples; this is particularly relevant in what concerns fixation.

We have been impressed by the frequent use in published papers and textbooks presenting ultrastructural aspects of mycobacteria of images of improperly fixed cells. Also, confusion is sometimes present in the interpretation of ultrastructural data, namely in the characterization of normal and altered cells. We think this is due to several reasons. On one hand, there is a rather generalized misunderstanding of what fixation is and of the effects of fixatives on biological materials. Additionally, some workers studying the micromorphology of bacteria, or using ultrastructural data as complementary information in experiments of several diverse kinds, are not aware of the fact that bacterial cells have specific requirements regarding fixation for electron microscopy.

In the present paper we discuss some points regarding the influence of fixation conditions on the ultrastructural pattern of mycobacterial cells, with special emphasis on the characterization of normal versus altered cells. This discussion is applicable, as well, to the related bacteria of the genus, *Nocardia*.

The ultrastructural pattern of normal mycobacterial cells

Using the experience gathered by our group as well as by others, we propose that the following micromorphological aspects are typical of a normal mycobacterial cell (that is, of cells growing *in vitro* or *in vivo*) when fixed by adequate procedures.

Cell envelopes. In normal cells they consist of a stratified cell wall and a cytoplasmic membrane (Figs. 1, 7a, and 7b). The inner layer of the cell wall, where the mucopeptide is located, is electron-dense (Figs. 1, 7a, and 7b, W_1) and is covered by an electron-transparent layer (Fig. 1 and 7a, W_2) which is sometimes difficult to visualize (Fig. 7b; see also Figs. 2b and 2c in ref. 21). Its visibility is improved either when a thin layer of irregular material covers its exterior surface (Figs. 1 and 7a, W_3) or when a bacillus is in close contact with another bacillus (Fig. 5)

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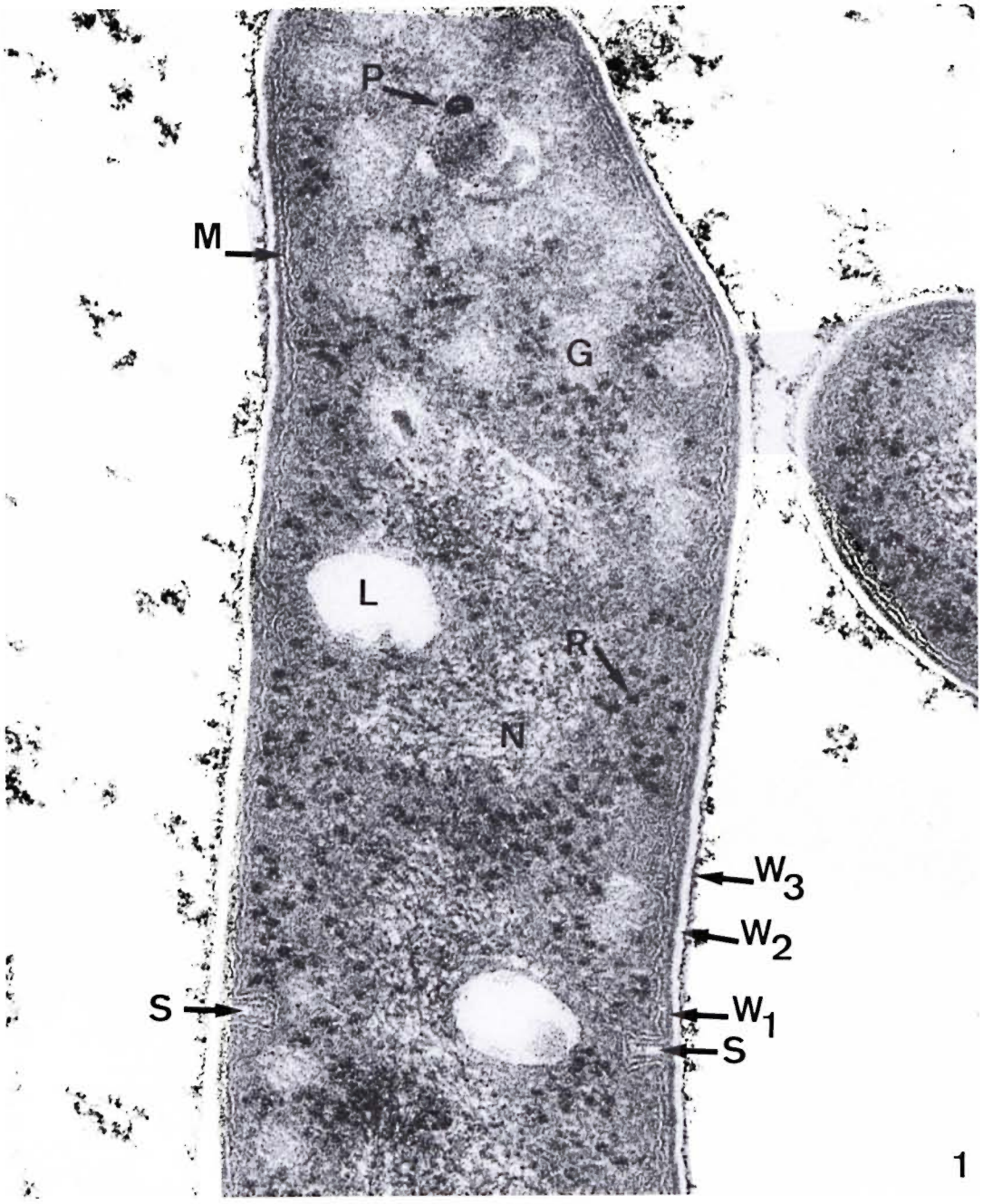

FIG. 1. Growing *M. aurum* (strain A$^+$); exponential culture in TB Broth (Difco) supplemented with 5% glycerol. Fixation with formaldehyde+glutaraldehyde+Ca^{++}-OsO_4-uranyl ([20,21]). Epon embedding. Section double-stained with uranyl and lead (×180,000).

M = cytoplasmic membrane
L = lipid inclusions
R = ribosomes
W_1, W_2, W_3 = layers of cell wall
P = polyphosphate inclusion
S = early stage of septum formation
N = nucleoid
G = polysaccharide (glycogen) inclusions, appearing as light areas

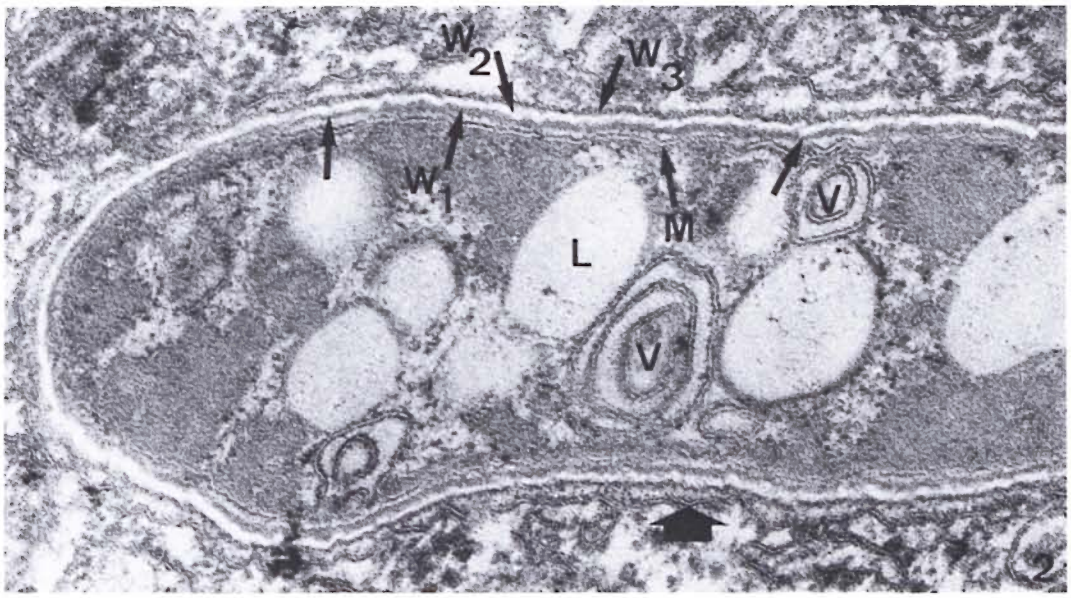

FIG. 2. Altered *M. tuberculosis* (H37Ra) inside a phagocytic vacuole of a mouse peritoneal macrophage. Four days after the intraperitoneal injection the bacillus is under degeneration. Notice the absence of ribosomes, the symmetric cytoplasmic membrane (M), the vesicules of concentric symmetric membranes (V), and the wavy mucopeptide layer of the wall (W_1) with zones with decreased thickness (unlabelled arrows). Fixation, embedding, and staining as in Figure 1 (×81,000).

or with dense structures. Both cell wall strata are continuous and without convolutions; breaks in the cell wall, deformations of its contour, or reduction in its thickness are signs of wall damage typical of autolysis or heterolysis (Figs. 2, 3, and 7e, and Fig. 5c in ref. 21). The cytoplasmic membrane has the triple-layered profile characteristic of biomembranes. Except in the case of *M. leprae* which have a peculiar membrane with a symmetric geometry ([20,21,22]) (Fig. 7b), the normal mycobacterial membrane, as in all other Gram-positives studied so far ([15,17]), has an asymmetric profile with the outer layer (facing the cell wall) thicker and denser than the inner layer (facing the cytoplasm). Such an asymmetry is more marked in sections contrasted with lead alone (Fig. 7a) or after Thiéry's technique for polysaccharides ([9]) (Fig. 4); less asymmetry is exhibited by membranes in sections double-stained with uranyl and lead (Fig. 1). The cytoplasmic membrane of normal mycobacterial cells is continuous, without breaks, but in samples embedded in low viscosity plastics (for example, Spurr or Ultra Low Viscosity Resin, TAAB) those membranes sometimes exhibit localized breaks (Fig. 7b, insert); we think this is due to polymerization damage which usually is not a problem with Epon. Mesosomes of varying complexity and size may be present, depending on the fixation procedure used ([15,17,29]): with the Ryter-Kellenberger fixation ([11]) they are frequently complex and prominent; after the double fixation aldehyde-OsO_4, they are simple and small; with uranyl-OsO_4, they are absent. Mesosome-like structures, usually in the form of concentric vesicules of symmetric membranes, are frequently found in damaged bacteria (Figs. 2 and 5, V). Since these structures are a result of bacterial alteration, they occur regardless of the fixation procedure ([17,24,25,27,28,29]).

Cytoplasm. Distinct ribosomes with the typical dimensions of procaryotic ribosomes are present (Fig. 1, R). Their number per cell varies with the strain; they are numerous in the fast growers like *M. aurum* (Fig. 1), and scant in the slow growers like *M. leprae* (see Fig. 5 in ref. 20 and Fig. 2 in ref. 21).

Nucleoid. A distinct, relatively condensed, nucleoid made up of non-aggre-

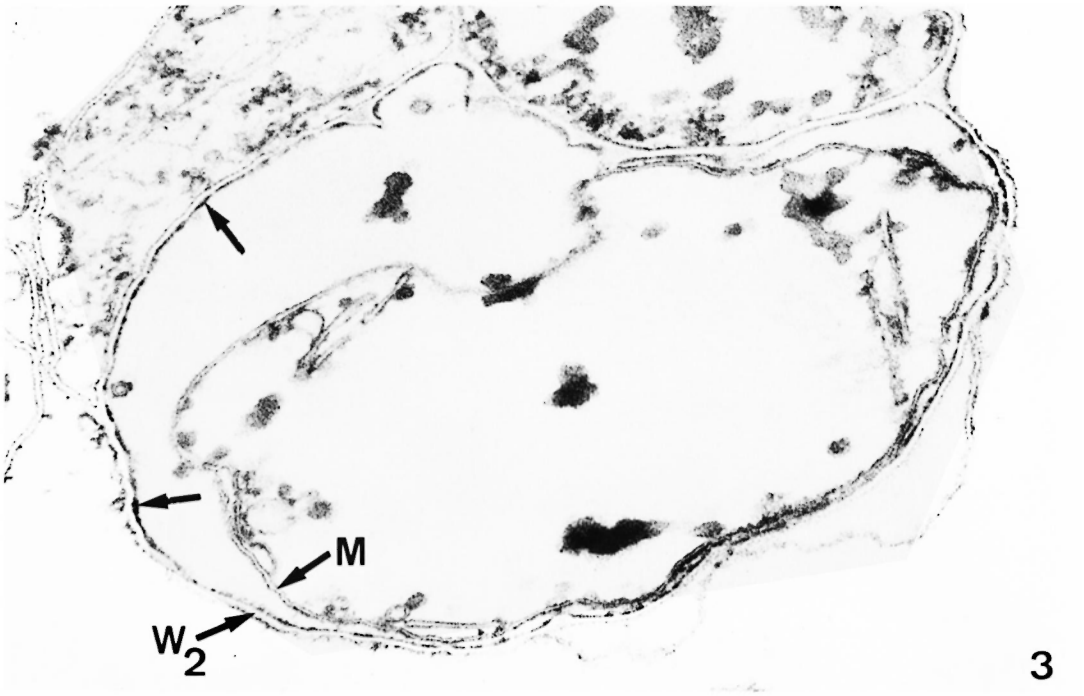

FIG. 3. Lysing *M. tuberculosis* (H37Rv) in an old culture in TB Broth (Difco) supplemented with 5% glycerol. Notice the symmetric cytoplasmic membrane (M) and the empty cell interior. The cell wall is distorted and its mucopeptide layer is extensively degraded, only some remnants of it being present (unlabelled arrows). The transparent layer of the wall is still present (W_2). Fixation, embedding, and staining as in Figure 1 (×80,000).

gated DNA fibrils is always present in longitudinal, central, sections (Fig. 1, N) but may, obviously, be missing in cross sections.

Inclusions. Polysaccharide (Figs. 1 and 4, G) and lipid (Figs. 1, 2, and 5, L) may be present. Their occurrence and amount are dependent on the physiological state of the bacteria ([1]). It is worth noting that polysaccharide inclusions appear as light areas (Fig. 1, G) in samples post-fixed with uranyl acetate ([10,32]). That the carbohydrate material is still there is demonstrated by staining with PAS-like reactions, such as with Thiéry's method ([31]) (Fig. 4). Polyphosphate inclusions may also be present (Fig. 1, P).

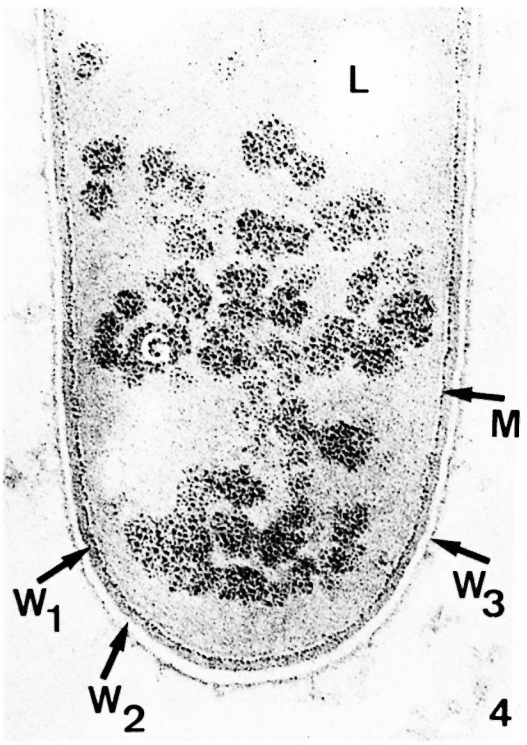

FIG. 4. *M. aurum* grown as indicated in Figure 1. Silver proteinate staining (Thiéry's reaction) ([31]) for polysaccharides. The glycogen areas (G) and the outer layer of the cytoplasmic membrane (M) exhibit positive reactions. Fixation and embedding as in Figure 1 (×80,000).

L = lipid inclusion
W_1, W_2, W_3 = layers of cell wall

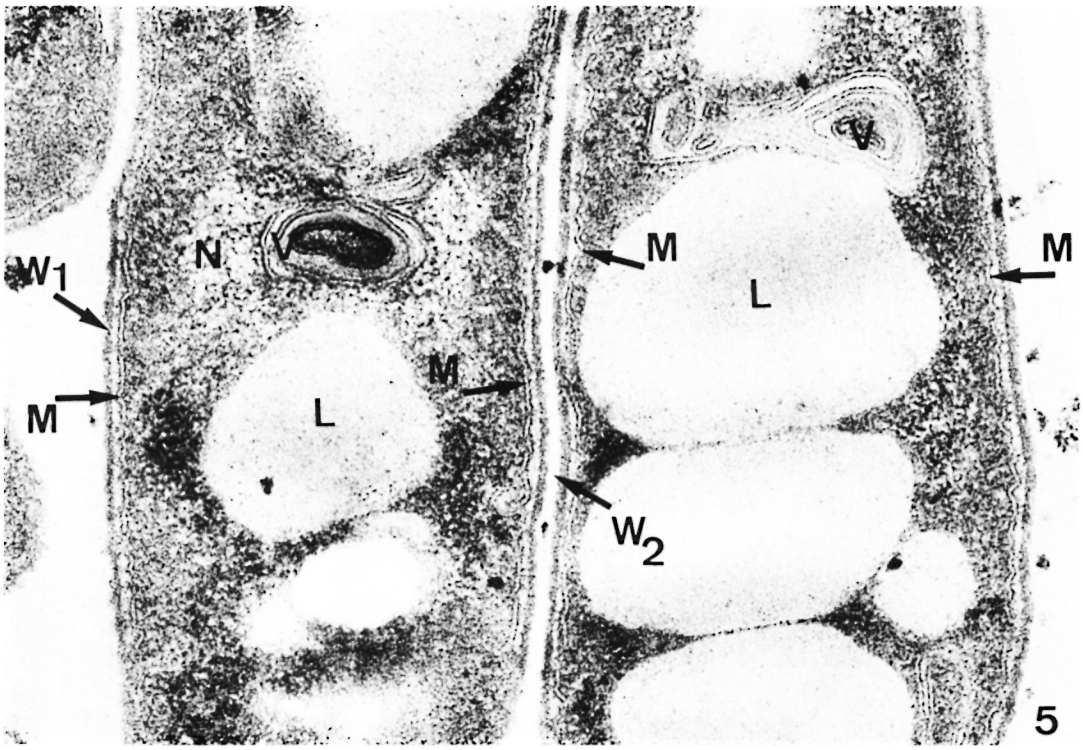

FIG. 5. Two *M. tuberculosis* (H37Ra) cells in close contact. Exponential culture (as in Fig. 1) exposed to 75 mM sodium azide for 48 hr. Notice the symmetric membranes (M), the vesicules of concentric symmetric membranes (V), and the absence of ribosomes. The clear space between the two apposed cells (W_2) corresponds to the electron-transparent layers of the walls of both cells. Fixation, embedding, and staining as in Figure 1 (×120,000).

L = lipid inclusions
N = nucleoid
W_1 = mucopeptide layer of cell wall

Cell division. Septum formation during cell division in mycobacteria is basically similar to that known to occur in most Gram-positives; however, and in contrast to what happens in *Bacillus* ([2]), the mucopeptide layer of the centripetally growing wall is double from the beginning in mycobacteria (Fig. 1, S; see also Fig. 9 in ref. 4). This feature of the septum formation seems to be due to the multi-layered structure of the wall of mycobacteria; it deserves a detailed study, including from the taxonomic point of view, since it may well be a micromorphological marker for acid-fast bacteria.

Influence of fixation conditions on mycobacterial ultrastructure

The characteristics described above for normal mycobacterial cells will be found only when the fixation procedure used in the preparation of the samples is adequate. Previous studies from this laboratory, as well as from others, on the effects of fixation conditions on the ultrastructure of Gram-positives ([11,15,17,18,20,23,29]) have led to the establishment of fixation conditions which we consider as suitable. We do not claim that the proposed methods are producing a perfect close-to-life preservation of all mycobacterial cell components; such a judgment would be, incidentally, very difficult to check. We do claim, however, that the proposed procedures give consistent results allowing the clear visualization of essential cellular components in all mycobacteria studied so far and producing a micromorphological pattern in normal cells that can be used as a standard for the characterization of altered cells. Moreover, there is a

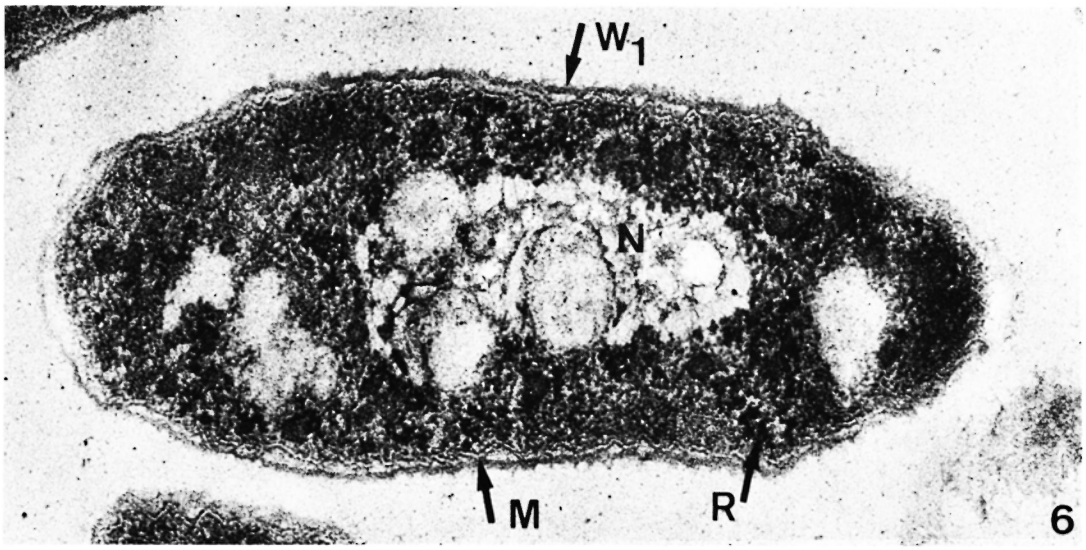

FIG. 6. *M. tuberculosis* (H37Ra) grown as indicated in Figure 1. Fixation with 1% OsO_4 buffered with 0.1 M phosphate, pH 7.0; no post-fixation with uranyl. Epon embedding, uranyl-lead staining. Notice the symmetric cytoplasmic membrane (M), the poor preservation of ribosomes (R) (compare to Fig. 1), and the coarsely aggregated DNA fibrils of the nucleoid (N) (×97,000).

W_1 = mucopeptide layer of cell wall

rationale behind the proposal of the fixation conditions to be described below; morphological, functional, and chemical considerations were utilized in the selection of such conditions, as reported and discussed elsewhere ([15, 17, 18, 23, 25, 29]).

In brief, the fixation conditions for the adequate preservation of mycobacteria are the following: Fixation should be carried out by fixatives containing appropriate concentrations of Ca^{++} [or Mg^{++} ([8])]; usually 10 mM is sufficient. When the fixation procedure uses a double fixation with aldehydes followed by OsO_4, both fixatives should be supplemented with the divalent cations. These cations increase the fixation rate ([7]) and stabilize the membranous structures ([15, 17, 23]). A post-fixation with uranyl ions (0.5–1.0% in water or in compatible buffer) ([16]) is advantageous, since uranyl, when used before dehydration, besides its contrasting effect, has a fixative action ([17, 18, 23, 29, 30]). When Ca^{++} (or Mg^{++}) is not present in appropriate concentrations during fixation, some significant alterations may result in the ultrastructural pattern of mycobacteria. This is particularly common when the prefixation step of the Ryter-Kellenberger procedure ([11]) is employed. This step consists of the addition of one volume of 1% OsO_4 fixative to 9–10 volumes of a bacterial suspension (usually a broth culture or a suspension in buffer). Consequently all components of the fixative are diluted tenfold, and the concentration of Ca^{++} and OsO_4 may become insufficient. This practice has deleterious effects on the preservation of Gram-positives, including mycobacteria, mainly when the bacteria are suspended in media with components which interfere with the fixative (see below). The alterations due to the inadequate concentrations (or absence) of divalent cations in the fixatives include ([11, 15, 17, 18, 20, 23, 29]): a) Poor preservation of the membranes which may appear with a symmetric profile in the strains which have normal membranes with asymmetric geometry (Figs. 6 and 7c, M); in some cases no membranes will be seen (Fig. 7d). b) Ribosomes may be absent or grossly disorganized (Fig. 6, R); this usually occurs in bacteria which also have poorly preserved membranes. c) DNA fibrils may appear aggregated (Fig. 6, N). It is important to mention that similar signs of poor fixation can be the result of the use of a suitable fixative under inadequate conditions. For example, Ca^{++} present in correct amounts in the fixative can be precipitated or chelated by

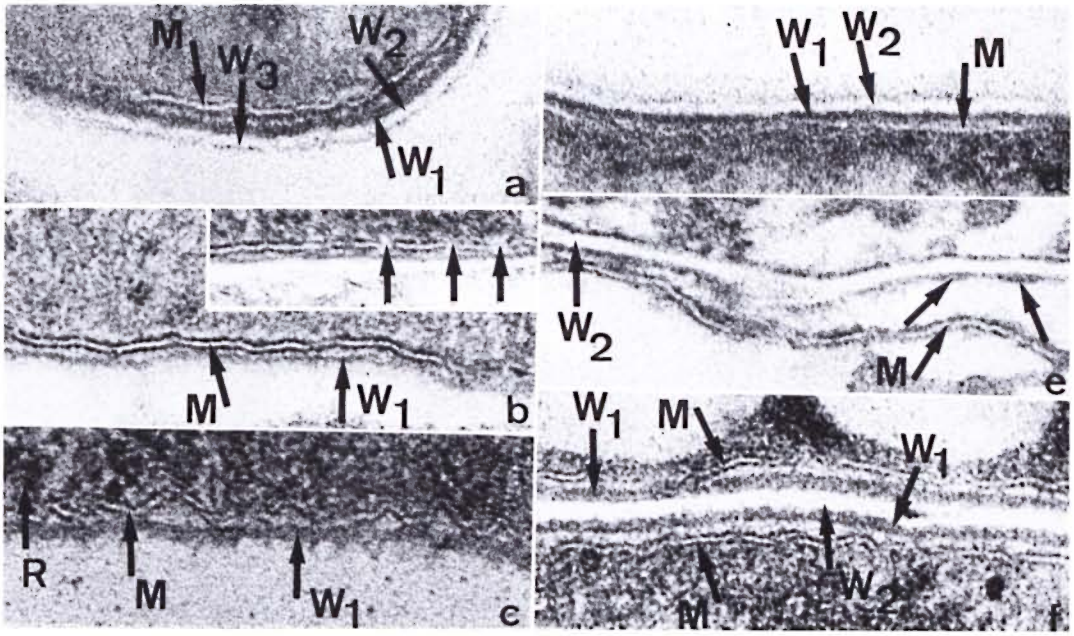

FIG. 7. High magnification (×167,000) of membrane profiles of mycobacterial cells under different conditions.

M = cytoplasmic membrane

W_1, W_2, W_3 = layers of the wall

a. Normal, *in vitro*-grown *M. tuberculosis* (H37Ra) fixed as in Figure 1. Lead staining. Notice the very asymmetric cytoplasmic membrane.

b. Normal *M. leprae* in the liver of experimentally infected armadillo. Notice the symmetric cytoplasmic membrane. Sample fixed, embedded, and stained as in Figure 1. Insert = same material except that it was embedded in Spurr resin. Notice the breaks in the membrane (unlabelled arrows).

c. Detail of Figure 6. Notice the symmetric and wavy profile of the membrane and the disorganized ribosomes (R).

d. Normal, *in vitro*-grown *M. tuberculosis* (H37Ra) fixed by the Ryter-Kellenberger procedure ([11]) (prefixation included). The OsO_4 fixative was not supplemented with Ca^{++} and the post-fixation with uranyl was not used. Notice the very poor preservation of the cytoplasmic membrane which is almost invisible (compare to Fig. 7a). In this section no ribosomes were visible. Epon embedding, uranyl-lead staining.

e. Detail of Figure 3. The cytoplasmic membrane is symmetric and the mucopeptide layer of the wall is extensively degraded, only remnants of it being left (unlabelled arrows).

f. Detail of Figure 5. Notice the symmetric profile of the membranes. The clear space between the two apposed cells (W_2) corresponds to the electron-transparent layers of both bacilli.

components of the medium in which the bacteria are suspended, mainly if the prefixation step is used, as discussed above; phosphate and carbonate will lead to such a result ([15,17,19]). Uranyl is neutralized by several buffers, including phosphate, carbonate, and cacodylate ([16]). Consequently, uranyl should not be used with those buffers, and if they are the vehicle for the OsO_4 fixative, an appropriate wash (with water or veronal-acetate, for example) must be included between the OsO_4 and uranyl fixations. Also, the concentration of OsO_4 can be dangerously reduced if the ratio fixative/bacterial mass is too low. The use of aldehydes as first fixatives is advantageous because they have fixative characteristics that complement those of OsO_4 and uranyl ([5,6]). We routinely use a mixture of 4% formaldehyde (prepared from paraformaldehyde) ([6]) and 1.25% glutaraldehyde in either 50 mM cacodylate buffer or 2.0% PIPES buffer ([12]), pH 7.0, supplemented with 10 mM $CaCl_2$. As discussed elsewhere ([20,21]), we chose this formula because formaldehyde is a fast penetrating fixative and glutaraldehyde has strong cross-linking capacity. Moreover, biopsies fixed in this mixture can

be processed for light microscopy for histological examination. The use of a primary fixative is a must whenever the samples cannot be processed for electron microscopy within 16–24 hr; this is the usual situation with biopsies.

A frequent source of poor fixation of bacteria inside tissues or cells can be explained by the aspects discussed above. We sometimes find in the literature images of poorly preserved mycobacteria inside naturally or experimentally infected hosts because the samples were simply fixed by procedures that were satisfactory for the tissues or cells but not for the bacteria [for example, phosphate buffered OsO_4 (Fig. 6)]. Whenever the ultrastructural study of mycobacteria concerns bacteria inside tissues or cells, a fixation procedure that fullfils the conditions outlined above for bacteria should be used. It is not difficult, incidentally, to preserve satisfactorily both the host cells and the bacteria. For example, the multiple procedure indicated above [formaldehyde+glutaraldehyde+Ca^{++}-OsO_4+Ca^{++}-uranyl ([20,21])] will do this, except that the glycogen inclusions will not be visualized as electron-dense rosettes due to the action of the uranyl acetate treatment ([32]), as already discussed.

Ultrastructural signs typical of damaged mycobacteria; importance of fixation conditions

As stressed above, the pattern we described for normal mycobacterial cells will be found only when an adequate fixation is used. A very important point is that incorrect fixation can lead to alterations in the ultrastructural pattern of normal bacteria that mimic those exhibited by cells altered by several processes like autolysis, heterolysis, antibacterial treatments, etc. The following are representative examples:

a) We have reported that in correctly fixed Gram-positives, including acid-fasts (but with the exception of *M. leprae*) ([20,21,22]) the occurrence of symmetric membranes is an early indication of membrane damage ([13,14,17,19,24,26,27,28]) (Figs. 2, 3, 5, 7e, and 7f, M). As discussed above, such a change in membrane profile can be the result of incorrect fixation of normal bacteria (Figs. 6 and 7c, M). In more advanced cell damage the membranes can be progressively solubilized until no membrane profiles are seen (Figs. 3, 4, 5, and 6 in ref. 21). Again, such a result can be due to improper fixation of normal membranes (Fig. 7d).

b) Mycobacterial cells exhibiting a compact cytoplasm but without ribosomes may be normal cells fixed by fixatives with insufficient concentrations of divalent cations and/or OsO_4 ([20]) (Figs. 6, 7c, and 7d) or altered cells when the fixation is adequate ([13,21,24,27]) (Figs. 2 and 5). Such a micromorphological pattern is sometimes the earliest indication of mycobacterial cell damage (usually associated with the presence of symmetric membranes) and it is not infrequent to see such cells characterized in the literature as intact.

Conclusions

The aspects discussed above are frequently ignored by mycobacteriologists dealing with ultrastructural aspects and are, obviously, important in the characterization of normal and altered mycobacterial cells under different conditions (for example, *in vitro* and *in vivo*), including for the characterization of species not previously studied, and in the description and interpretation of degenerative alterations occurring in mycobacteria under several conditions leading to degenerative processes; this is the case, among others, of mycobacterial cells affected in patients or infected animals by the treatment or by the host defensive mechanisms, and in studies on chemotherapeutic agents, mycobacterial cell autolysis, etc. In conclusion, mycobacteriologists working on the ultrastructure of mycobacteria or interpreting the images presented by others should be aware that:

a) Mycobacterial cells, like other bacteria, have specific requirements for fixation for electron microscopy.

b) When studying mycobacterial cells inside tissues, the fixation procedure must be directed to the adequate preservation of bacteria.

c) To correctly characterize normal and altered mycobacteria under the ultrastructural point of view, it is important to be sure that the fixation procedure is adequate or, at least, to be aware of what the fixation conditions were and what effects such conditions have upon bacterial ultrastructure. The fact that improperly preserved bacterial cells frequently exhibit ultrastructural alter-

ations identical to those of damaged bacteria is very important in this context.

SUMMARY

In the present report the authors discuss several aspects of the ultrastructure of mycobacterial cells as seen by transmission electron microscopy of ultrathin sections that are relevant in the characterization of normal versus altered bacteria. The importance of the use of adequate fixation conditions is stressed and illustrated with examples showing that normal, but inadequately fixed, mycobacterial cells may exhibit micromorphological alterations similar to those typical of cells affected in several situations such as autolysis, heterolysis, and antibacterial treatments.

RESUMEN

En el presente trabajo los autores discuten varios aspectos de la ultraestructura de las células micobacterianas según se ven por microscopía electrónica en cortes ultradelgados. Estos aspectos ultraestructurales son relevantes en la caracterización de bacterias normales y de bacterias alteradas. La importancia de las condiciones apropiadas para la fijación de los especímenes se ilustra con ejemplos que demuestran que las células micobacterianas normales pero inadecuadamente fijadas pueden exhibir alteraciones morfológicas similares a las observadas en células alteradas por heterolisis, por autolisis, o por tratamiento antibacteriano.

RÉSUMÉ

Dans cet article, les auteurs passent en revue plusieurs aspects de la structure fine des cellules mycobactériennes, qui ont trait à la mise en évidence des caractéristiques des bacilles normaux par comparaison avec les bacilles endommagés. Cette étude a été menée par microscopie électronique de sections ultra-minces. On souligne l'importance de recourir à des conditions de fixation adéquates; ceci est illustré par des exemples qui montrent que des cellules mycobactériennes normales, mais mal fixées, peuvent montrer des altérations micro-morphologiques similaires à celles qui caractérisent les cellules endommagées dans des conditions telles que l'autolyse, l'hétérolyse, et les traitements antibactériens.

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