

## New Data on the Ultrastructure of the Membrane of *Mycobacterium leprae*<sup>1</sup>

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We have reported before on the micro-morphology of *Mycobacterium leprae* in samples from natural and experimental hosts using transmission electron microscopy of ultrathin sections after fixation by a procedure that was found to adequately preserve the ultrastructure of cultivable mycobacteria (<sup>9, 11</sup>). That procedure consists of a prefixation with glutaraldehyde plus formaldehyde plus calcium, followed by a fixation with OsO<sub>4</sub> plus calcium and a postfixation with uranyl acetate (GaFaCa-Os-U). This fixation protocol proved quite useful for the handling of leprosy biopsies because they often have to be sent to a distant electron-microscopy laboratory for processing and aldehyde fixatives are much better suited for this purpose than osmium tetroxide fixatives. In samples of skin biopsies of lepromatous leprosy patients; the livers, spleens, lymph nodes, and lepromas of armadillos with natural or experimental leprosy; skin biopsies from monkeys with experimental leprosy; and the foot pads of nude mice with experimental leprosy processed for electron microscopy immediately after collection and using the above fixation procedure, continuous membranes of normal-looking *M. leprae* showed a symmetric profile after lead staining and a symmetric Thiéry reaction (<sup>21</sup>), indicating the presence of periodic acid-Schiff (PAS)-positive components in both membrane layers (<sup>7, 11-13, 15</sup>). Symmetric membranes were also found in *M. leprae* from fresh skin lepromas from lepromatous leprosy patients after fixation with 4% OsO<sub>4</sub> in water supplemented or not with CaCl<sub>2</sub>(<sup>9</sup>) and in -70°C-stored skin lepromas and liver from an armadillo with experimental leprosy after fixation with

buffered 1% OsO<sub>4</sub> supplemented with several membrane stabilizing divalent cations (<sup>10</sup>). These observations confirmed the initial finding that no asymmetric membranes could be found in *M. leprae*, the symmetric profile appearing as the image of the membrane in otherwise normal-looking bacilli.

Such a conclusion, however, raises important questions which were stressed in our previous publications (<sup>13, 16</sup>). Namely, a) *M. leprae* membrane symmetry is in contrast with the asymmetry found in all other normal gram-positive bacteria, including mycobacteria and nocardiae, studied until now, which have membranes with the outer layer denser than the inner layer and PAS-positive components exclusively in the outer layer (<sup>6, 7, 9, 11, 13</sup>), and b) it is difficult to accept, because of well-established concepts on bacterial membrane structure and physiology (<sup>7, 23</sup>), that the membrane of normal *M. leprae* has PAS-positive components in both layers. A symmetric distribution of PAS-positive components in the membrane of *M. leprae* would represent a striking and unique exception to the concept that the membranes of gram-positive bacteria have amphiphilic molecules, like polysaccharides or equivalent charged polymers, exclusively in the outer layer (<sup>7, 23</sup>) and, as a consequence, an asymmetric geometry after lead or Thiéry stainings (<sup>7</sup>). We, therefore, admitted that the symmetric *M. leprae* membranes seen in the samples we studied would correspond to abnormal membranes (<sup>13, 16</sup>). The idea that the normal membrane of *M. leprae* would be asymmetric, like that of other mycobacteria, was strengthened by the recent finding of a few *M. leprae* cells with asymmetric membranes in a nude mouse with experimental leprosy (<sup>16</sup>).

We tested the possibility of the abnormal symmetric geometry of the membranes found in otherwise normal-looking *M. leprae* being the result of a deficient membrane preservation by the GaFaCa-Os-U fixation protocol or of the use of frozen samples for

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the ultrastructural studies. Such a possibility was confirmed. In the present study, we report in detail the results regarding the detrimental effects of aldehyde prefixation on the ultrastructure of the *M. leprae* membrane and preliminary data on the alterations induced in that membrane by freezing and thawing.

#### MATERIALS AND METHODS

**Mycobacteria.** *M. leprae* was studied: a) in samples from armadillos 1177, 1210, and 1485 collected in southwestern Louisiana, U.S.A., in cooperation with Dr. G. P. Walsh (Armed Forces Institute of Pathology, Washington, D.C., U.S.A.). The animals had naturally acquired leprosy detected by microbiological and histopathological studies carried out by Drs. G. P. Walsh, W. M. Meyers, and C. H. Binford (Armed Forces Institute of Pathology, Washington, D.C., U.S.A.). The samples included skin lepromas, lymph nodes, livers, and spleens; b) in one foot pad from the nude mouse 790-32 with experimental leprosy supplied by Dr. R. C. Hastings (GWL Hansen's Disease Center, Carville, Louisiana, U.S.A.); c) in skin biopsies from two patients with lepromatous leprosy—patient A.F., biopsy supplied by Prof. J. Mesquita Guimarães, Department of Dermatology, School of Medicine, Porto, Portugal, and patient M.S., biopsy supplied by Prof. A. Poiars Batista, Department of Dermatology, School of Medicine, Coimbra, Portugal.

*M. lepraemurium* (Douglas strain) and *M. avium* (strain ATCC 25291) were inoculated ( $2 \times 10^8$  bacilli) by the intraperitoneal route into C57BL/6 mice and the livers were collected after 4–5 months. *M. aurum* (strain 14120005 from the collection of the Institut Pasteur, Paris) was grown in Nutrient Broth (Difco) supplemented with 0.04% Tween 80 at 37°C for 2 days, with occasional shaking. *M. avium* (same strain as above) and *M. tuberculosis* H37Ra (strain NCTC 7417) were grown in 7H9 Broth (Difco) supplemented with 0.04% (v/v) Tween 80 at 37°C, with occasional shaking, for 6 days. As inocula, exponential cultures in the same medium were used. The bacteria were collected from the broth cultures by centrifugation ( $2000 \times g \times 10$  min), and the pellets processed for electron microscopy.

**Electron microscopy.** Two fixation procedures were applied to all samples: a) the previously reported method using aldehyde prefixation<sup>(9, 11)</sup> [GaFaCa (1 to several days)-OsO<sub>4</sub> (16–24 hr)-uranyl (1 hr)]; b) OsO<sub>4</sub> (1% in Palade's veronal acetate buffer, pH 6.0, supplemented with 10 mM calcium<sup>(4)</sup>), for 16–24 hr, then washed in several changes of 50 mM cacodylate buffer supplemented with 10 mM Ca<sup>2+</sup>, pH 7.0; fixation with GaFaCa<sup>(9)</sup> for 24 hr; after a wash in water, the samples were postfixed in aqueous 1% uranyl acetate for 1 hr (Os-GaFaCa-U). All fixations were carried out at room temperature. Since we have found that freezing and thawing of *M. leprae* induces membrane alterations<sup>(17)</sup>, in the present study all samples were fixed within minutes after collection. For comparison, homogenates of the armadillo samples (made in Dubos-Middlebrook liquid medium) were also fixed after one freezing-thawing cycle (–70°C/room temperature). Further processing for electron microscopy was carried out with ethanol dehydration and Epon embedding. Ultrathin sections were cut with an LKB Ultratome III and contrasted with lead citrate<sup>(22)</sup> for 5 min or stained by the procedure of Thiéry<sup>(21)</sup> for detection of PAS-positive molecules as modified by us<sup>(12)</sup>. Observations were done with a Siemens' Elmiskop 1A or 102 electron microscope. Tracings of membrane profiles were made on photographic negatives with a Joyce-Loebl MK III CS microdensitometer set to an arm ratio of 50 × and a slit of 0.5 nm.

**Terminology of membrane profiles after Thiéry staining.** We use here the Thiéry staining method<sup>(21)</sup> to cytochemically label PAS-positive molecules in ultrathin sections. It has been shown that the Thiéry method is most useful for the detection of the surface amphiphiles in acid-fast bacteria<sup>(2, 3, 7, 8, 11–14, 16)</sup>. Nevertheless, caution is required in the interpretation of the Thiéry labeling seen on bacterial membranes. In brief, we recall now the prerequisites for the accurate interpretation of the technique. A positive Thiéry staining of bacterial membranes consists of the presence of localized black dots; a light staining usually occurs in membrane layers with a negative Thiéry reaction due to the nonspecific staining prop-

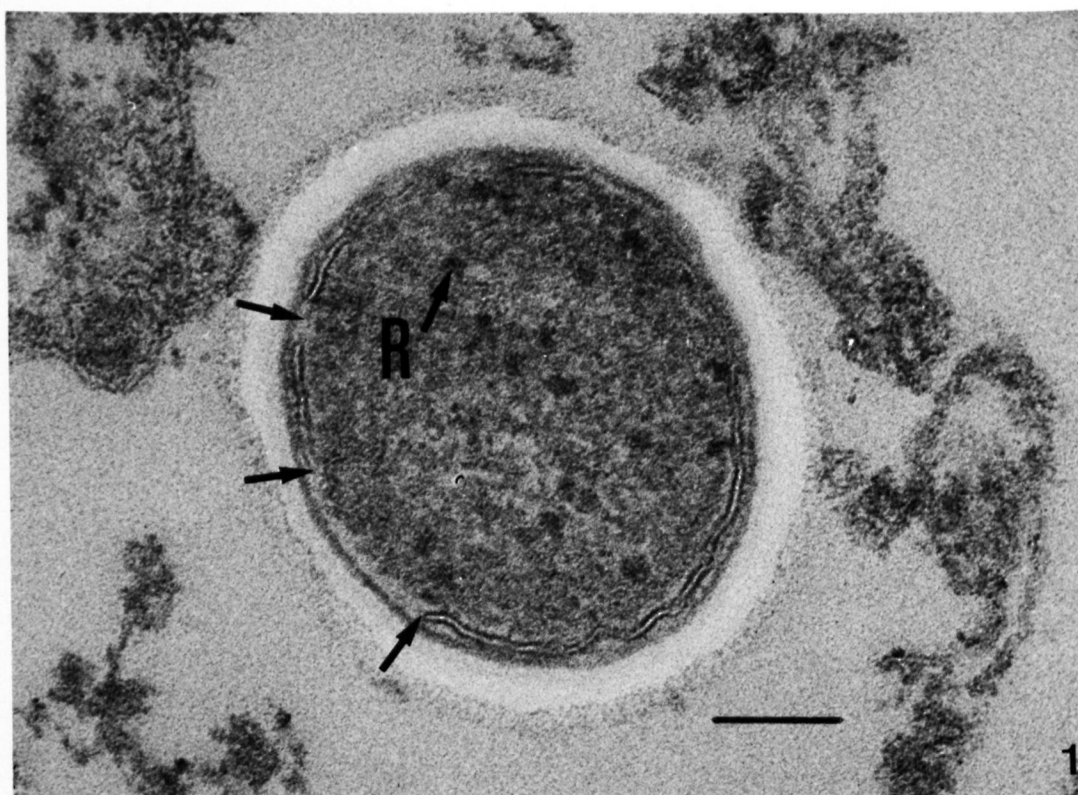


FIG. 1. Ultrastructure of the membrane of *M. leprae* in a skin leproma from armadillo 1210 after immediate fixation with GaFaCa-Os-U (lead staining). Note symmetric cytoplasmic membrane with discontinuities (unlabeled arrows) and the presence of ribosomes (R). (Bar = 100 nm.)

erty of silver proteinates. The membranes of normal gram-positive bacteria give a PAS-positive reaction exclusively in the outer membrane layer, indicating an absolute asymmetry in the distribution of the so-called surface amphiphiles (<sup>7, 23</sup>). Similarly,

in acid-fast organisms the Thiéry staining of normal membranes reveals the presence of PAS-positive components also restricted to the outer layer (<sup>2, 3, 7, 8, 11-14</sup>). In gram-positive bacteria, including acid-fast bacteria, under degradation both *in vitro* and *in vivo*

TABLE 1. Ultrastructural quantitative analysis of the membranes of *M. leprae* in tissues from armadillos and of *M. lepraemurium* and *M. avium* in tissues from mice (sections stained by lead citrate).

Bacterium	Sample	Fixation	Membrane profile		Total <sup>a</sup>
			Asymmetric	Symmetric	
<i>M. leprae</i>	Fresh	Os-GaFaCa-U	90.7%	9.3% <sup>b</sup>	315
		GaFaCa-Os-U	0.7%	99.3% <sup>c</sup>	469
	Frozen	Os-GaFaCa-U	24.7%	75.3% <sup>d</sup>	93
<i>M. lepraemurium</i>	Fresh	Os-GaFaCa-U	99.4%	0.6% <sup>b</sup>	158
		GaFaCa-Os-U	95.4%	4.6%	153
<i>M. avium</i>	Fresh	Os-GaFaCa-U	96.1%	3.9% <sup>b</sup>	154
		GaFaCa-Os-U	94.5%	5.5%	180

<sup>a</sup> Total bacillary profiles with membranes.

<sup>b</sup> 100% of the bacilli with these membranes were without ribosomes.

<sup>c</sup> 73.9% of these membranes were discontinuous and 81.3% of the bacilli had ribosomes.

<sup>d</sup> 51.4% of these membranes were discontinuous and 76.2% of the bacilli had ribosomes.

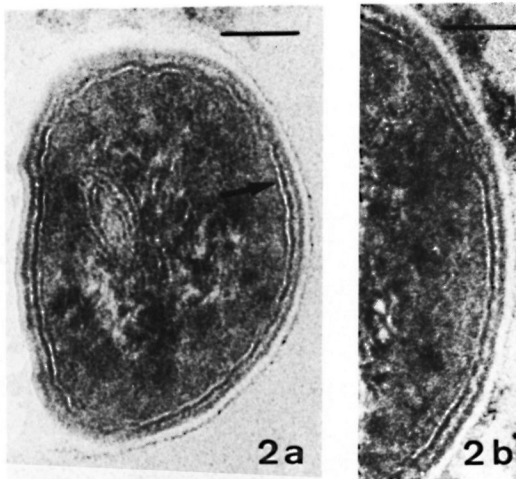


FIG. 2. Ultrastructure of the asymmetric membrane of *M. leprae* in samples fixed with Os-GaFaCa-U (lead staining). (Bars = 100 nm.)

**a** = One bacillus from a skin leproma of armadillo 1210. Outer membrane layer is thicker and more electron-dense than inner layer.

**b** = Sample from nude mouse 790-32. Membrane profile is identical to that in Fig. 2a.

the membrane geometry of lead-stained cells changes from asymmetric to symmetric<sup>(6, 7)</sup>, and the PAS-positive molecules are lost from the outer layer, that is, the membrane becomes PAS-negative<sup>(7, 8, 13)</sup>. In normal bacteria, the membrane profile is always continuous; sometimes, due to undulations in the membrane, the profile may erroneously look interrupted. This must not be confused with true interruptions of the membrane that can occur in bacteria under *in vitro* or *in vivo* degradation<sup>(6-8)</sup>.

Three additional membrane patterns are described here for Thiéry-stained mycobacterial membranes primarily fixed with aldehydes: 1) An intermediate pattern characterized by PAS-positive molecules on both membrane layers with predominance in the outer layer; these membranes are difficult to distinguish from the symmetric membranes in lead-stained sections. 2) A symmetric pattern characterized by PAS-positive molecules in equal amounts in both membrane layers. This pattern can assume two subtypes: a) Heavy staining of both layers (i.e., the inner layer shows a Thiéry reaction identical to that seen in the outer layer of normal asymmetric-stained profiles); this type of staining is frequently seen

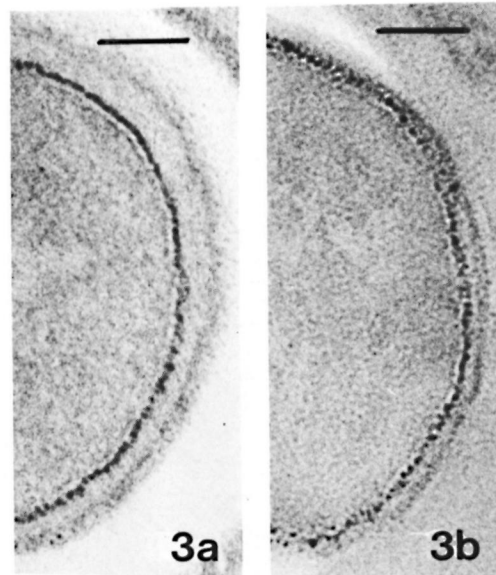


FIG. 3. **a** = Same sample as in Fig. 2a. Thiéry staining revealing PAS-positive components only in outer layer of the membrane. (Bar = 100 nm.)

**b** = Same sample as in Fig. 2b. Thiéry staining, showing same pattern of distribution of PAS-positive components as in Fig. 3a. (Bar = 100 nm.)

in discontinuous membranes. b) Light staining of both layers (i.e., the staining intensity of both layers is weaker than that of the outer layer of the normal asymmetric membranes); this type of staining is usually found in continuous membranes. In lead-stained sections these two subtypes of membranes appear as symmetric profiles with, respectively, a strong or a light staining of both layers.

## RESULTS

As is usual with mycobacterial populations in infected hosts<sup>(15, 16)</sup>, normal-looking and degenerating bacilli were found in the samples studied. In each sample, the percentage of bacilli with membranes was found to be unaffected by the fixation procedures studied. The quantitative ultrastructural analysis of mycobacterial membranes was, therefore, carried out by scoring only bacillary profiles having membranes.

**Membrane ultrastructure after immediate fixation.** The ultrastructure of the *M. leprae* membranes in all samples studied was drastically affected by the fixation conditions used (Table 1). Confirming our previous ob-

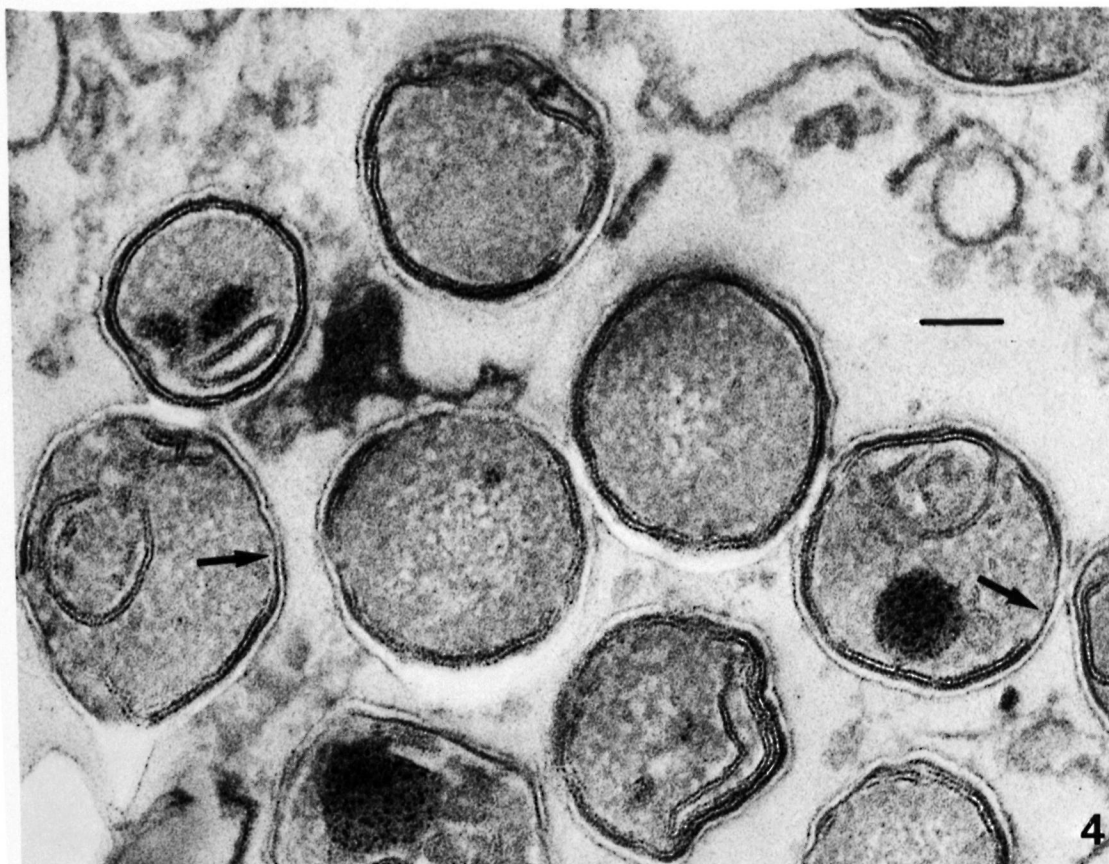


FIG. 4. Thiéry staining of a skin leproma from armadillo 1210, after immediate fixation with GaFaCa-Os-U, showing symmetric membranes with PAS-positive components in both layers. Several membranes have gross discontinuities (unlabeled arrows). (Bar = 100 nm).

servations<sup>(9-13, 15)</sup>, after fixation with GaFaCa-Os-U and lead staining almost all bacilli with membranes had symmetric membranes. Many of these symmetric membranes were discontinuous and were present in bacilli with ribosomes (Fig. 1). On the other hand, in samples fixed by Os-GaFaCa-U most of the bacteria with membranes had continuous, asymmetric membranes and ribosomes (Figs. 2a and 2b). The use of the Thiéry staining method showed that the asymmetric membranes in samples fixed with Os-GaFaCa-U had PAS-positive components exclusively located in the outer layer (Figs. 3a and 3b), indicating an absolute asymmetry in the distribution of PAS-positive molecules. In these samples no membranes were found with a symmetric Thiéry staining. In contrast, many symmetric membranes (84.5% of total membranes in armadillo samples) present after

fixation with GaFaCa-Os-U showed a strong staining for PAS-positive molecules in both layers; 71.7% of these membranes had gross discontinuities (Fig. 4). It is likely that the actual percentage of bacilli with discontinuous membranes is higher than the value found, because membranes that are continuous in a section may be discontinuous in another plane of sectioning. To clarify this point the study of serial sections is necessary. The use of the Thiéry technique also showed that 3.9% of the bacilli in samples fixed by GaFaCa-Os-U had the intermediate staining pattern.

The results in Table 1 represent the cumulative values obtained in a quantitative study of samples from armadillos 1210 (skin leproma, liver, and axillary lymph node) and 1177 (liver). In all of the samples studied, the above reported patterns of *M. leprae* membranes in relation to the fixation con-

ditions were observed, indicating that it is a phenomenon common to all samples studied.

Measurements of the membrane thickness were made on microdensitometric tracings of membrane profiles (Fig. 5a). The mean peak-to-peak distance found for the asymmetric membrane present in lead-stained sections of samples fixed with Os-GaFaCa-U was  $6.49 \pm 0.36$  nm ( $N = 30$ ) (Table 2). This value is significantly lower than that previously reported for the symmetric membranes present in samples primarily fixed by GaFaCa-Os-U (<sup>12, 13</sup>). This is because the inner layer of the symmetric membranes has PAS-positive components that increase its electron density with the displacement of the inner peak to the cytoplasmic side of the membrane profile (Fig. 5b).

No quantitatively important differences were found in the membrane ultrastructure in the *in vivo*-grown cultivable mycobacteria studied with the two fixation protocols. In Table 1 the results regarding *M. lepraemurium* and *M. avium* are presented. Most bacteria have asymmetric membranes with Thiéry-positive components exclusively in the outer layer (Fig. 6a) regardless of the fixation procedure. In samples fixed by GaFaCa-Os-U, 1.9% and 1.6% of the membranes showed the intermediate Thiéry staining in *M. lepraemurium* and *M. avium*, respectively (Fig. 6b). Moreover, and contrasting with what was found with *M. leprae*, in *M. lepraemurium* and *M. avium* primarily fixed with aldehydes only 2.0% and 3.8%, respectively, of the membranes

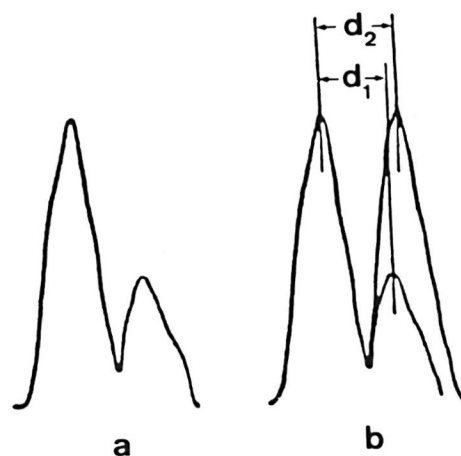


FIG. 5. **a** = Microdensitometric tracing of the membrane of *M. leprae* fixed with Os-GaFaCa-U and stained with lead. Tracing was made at zone indicated by the arrowhead in Fig. 2a. Outer layer of the membrane is represented by peak on the left; membrane geometry is clearly asymmetric.

**b** = Asymmetric tracing from Fig. 5a was superimposed on a symmetric tracing constructed by repeating in the inner side of the profile of the outer side from Fig. 5a. Peak-to-peak distance in the asymmetric tracing ( $d_1$ ) is about 10% less than peak-to-peak distance in the symmetric tracing ( $d_2$ ). Such a difference is identical to the difference between the mean distances found for the asymmetric and symmetric membranes (Table 2). Same result is obtained by superimposing the asymmetric tracing to a symmetric one from membranes fixed with GaFaCa-Os-U.

showed a symmetric Thiéry staining. In these membranes the intensity of staining of the two membrane layers was lighter than that seen in the outer layer of the asym-

TABLE 2. Peak-to-peak distances from microdensitometric tracings of mycobacterial membranes in lead-stained Epon sections of samples from infected armadillos (*M. leprae*) or mice (*M. lepraemurium* and *M. avium*).

Bacterium	Fixation	Membrane profile	No. measurements	Peak-to-peak distance and S.D. (nm) <sup>a</sup>
<i>M. leprae</i>	GaFaCa-Os-U	Symmetric	90	$7.05 \pm 0.50^b$
	Os-GaFaCa-U	Asymmetric	30	$6.49 \pm 0.36$
<i>M. lepraemurium</i>	GaFaCa-Os-U	Asymmetric	33	$6.45 \pm 0.23^b$
	Os-GaFaCa-U	Asymmetric	27	$6.44 \pm 0.49$
<i>M. tuberculosis</i> H37Ra	GaFaCa-Os-U	Asymmetric	12	$6.48 \pm 0.45^b$

<sup>a</sup> Statistical analysis (Student's *t* test) of the values for the peak-to-peak distances showed that the difference between the thickness of the asymmetric membrane of *M. leprae* (after fixation with Os-GaFaCa-U) and those of *M. lepraemurium* and *M. tuberculosis* H37Ra (irrespective of the fixation protocol) is not significant ( $p > 0.05$ ).

<sup>b</sup> Values taken from Silva and Macedo (<sup>13</sup>).

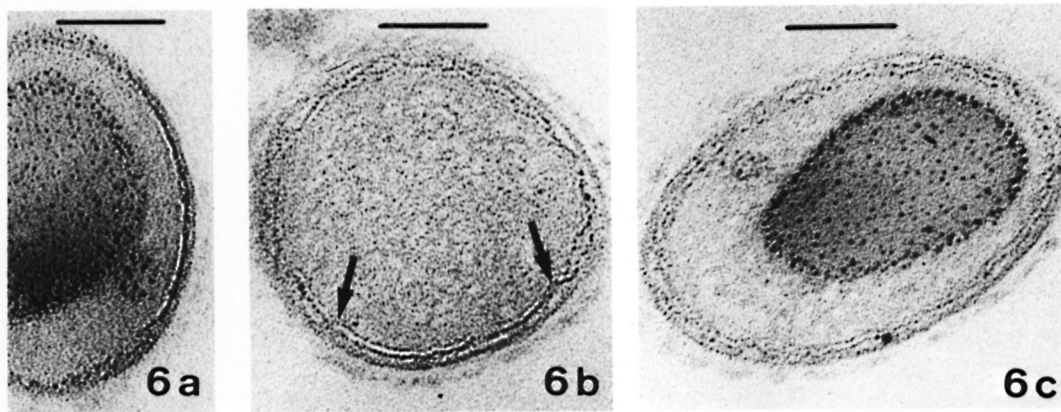


FIG. 6. Thiéry staining of *M. avium* fixed with GaFaCa-Os-U. (Bar = 100 nm.)

a = Bacillus with a fully asymmetric membrane.

b = Intermediate staining clearly seen at the zone where membrane is cut perpendicularly (between arrows); note inner membrane layer is lightly positive and outer layer has a positive staining that is weaker than that in Fig. 6a.

c = Symmetric, light staining in both membrane layers; note positive reaction is approximately identical in both layers and is lighter than that in outer layer of asymmetric profile in Fig. 6a.

metric membranes (Fig. 6c). No discontinuous membranes with this staining pattern were found. In *M. lepraemurium* and *M. avium* no membranes were found with a strong Thiéry staining of both layers.

The mean peak-to-peak distance found for the asymmetric membranes of *M. lepraemurium* present in the lead-stained sections of these samples fixed with Os-Ga-FaCa-U was  $6.44 \pm 0.49$  nm (N = 27) (Table 2). Similar results were obtained with *M. aurum* and *M. tuberculosis* H37Ra in mice, and with *in vitro*-grown *M. avium*, *M. aurum*, and *M. tuberculosis* H37Ra (not shown). These results confirm previous data showing that fixation with GaFaCa-Os-U is adequate for several cultivable mycobacteria (7, 11).

**Ultrastructure of *M. leprae* membrane after freezing and thawing.** We have previously found that freezing and thawing of *M. leprae* cells induces membrane alterations (17), and a detailed report on this subject is in preparation. For the purpose of the present paper, it suffices to report that after one freezing-thawing cycle the proportion of cells with symmetric membranes seen in lead-stained sections of samples fixed by Os-Ga-FaCa-U was 75.3% (Table 1); 76.2% of the bacilli with symmetric membranes had ribosomes. The vast majority of those symmetric membranes had PAS-positive com-

ponents in both layers and 51.4% were fragmented.

## DISCUSSION

Taking into consideration well established concepts on membrane structure and physiology (7, 23) referred to earlier in this paper, we propose that the asymmetric profile of *M. leprae* membrane now found in fresh samples primarily fixed with buffered and calcium-supplemented osmium tetroxide is a better morphological expression of the membrane structure of the leprosy bacillus than the symmetric profile present in samples primarily fixed by aldehydes. We consider that the two ultrastructural aspects of the membrane of *M. leprae* seen with the two fixation protocols used in the present study are due to the peculiar sensibility of the membrane to aldehydes, which induce an artifactual symmetric image. This interpretation is based on the observation that many *M. leprae* cells found in samples fixed with GaFaCa-Os-U had symmetric membranes with gross discontinuities but still contained distinct ribosomes; such membranes exhibited PAS-positive components in both layers. The first observation is difficult to reconcile with previous data showing that in gram-positive bacteria under *in vitro* degradation due to cell lysis or to treatments with membrane-active agents, the

membrane alterations are accompanied by ribosome disappearance<sup>(5, 18-20)</sup> and that mycobacteria degenerating *in vivo* due to the macrophages' antimicrobial activity have early membrane alterations that are, as well, always accompanied by ribosome disintegration<sup>(8)</sup>. Moreover, if the symmetric, discontinuous membranes were membranes altered by a degradative process occurring in the host, it is likely that they would have lost their PAS-positive components, as was found with several mycobacteria previously studied<sup>(8, 13)</sup>. The interpretation that the symmetric membranes present in aldehyde prefixed samples are artifactual is further supported by preliminary studies that used a different approach. We have been using the fluorescein diacetate-ethidium bromide staining method of Kvach, *et al.*<sup>(1)</sup> in parallel with the quantitative ultrastructural analysis of *M. leprae* populations in armadillo samples. With the above fluorescence staining, only the bacteria with damaged membranes stain red because the presence of a normal membrane prevents the access of ethidium bromide to the cell interior. We found that in a given sample the percentage of *M. leprae* cells showing red fluorescence is much lower than the percentage of cells with membrane ultrastructural abnormalities seen with GaFaCa-Os-U fixation.

The observation that the freezing and thawing of *M. leprae* induces membrane alterations similar to those produced by aldehyde prefixation<sup>(13)</sup> and Table 1) explains our previous finding<sup>(10)</sup> of symmetric membranes in *M. leprae* in samples from skin lepromas and the liver of an armadillo with experimental leprosy that were stored at  $-70^{\circ}\text{C}$  before fixation with buffered and calcium-supplemented osmium tetroxide.

The results now presented confirm our previous contention that the symmetric membranes we found in *M. leprae* would be abnormal<sup>(13, 16)</sup>, and furnish the additional information that such abnormality derives from the fixation technique or from the use of frozen samples.

The membrane of normal *M. leprae* is not, therefore, an exception to the general concept that membranes of all normal gram-positive bacteria have PAS-positive components exclusively located in the outer layer<sup>(7, 23)</sup> and, as a consequence, an asymmetric profile in lead- or Thiéry-stained sections

(7). The thickness of the asymmetric membrane now described in *M. leprae* is not significantly different from that of *in vivo*-grown *M. lepraemurium* and *M. tuberculosis* H37Ra. The present results show, however, that the membrane of the leprosy bacillus is different from the membrane of the other mycobacteria studied in regard to its sensitivity to aldehyde fixatives.

The here-reported occurrence, in *M. leprae*, *M. lepraemurium*, and *M. avium* fixed with GaFaCa-Os-U, of a few membranes with a light Thiéry staining of the inner layer suggests the occurrence of a rearrangement of the membrane structure induced by the aldehydes. Since the membranes with the light Thiéry staining of the inner layer had a lower intensity of staining of the outer layer as compared to the membranes with absolute asymmetry of Thiéry staining, it is likely that the rearrangement of the membrane structure induced by the aldehydes involves the displacement of PAS-positive molecules from the outer to the inner layer. The reported occurrence in *M. lepraemurium* and *M. avium* fixed by GaFaCa-Os-U of continuous membranes with a light, symmetric Thiéry staining of both layers is likely to result, as well, from the displacement of PAS-positive molecules from the outer to the inner layer. Therefore, the membranes with PAS-positive molecules in both layers, but with a stronger reaction in the outer layer, would correspond to an intermediate step in the change from fully asymmetric to fully symmetric. In *M. leprae*, but not in *M. lepraemurium* and *M. avium*, the final step in the membrane alteration induced by aldehydes is characterized by the production of discontinuous membranes with a symmetric, strong staining of both layers. This observation suggests that different mechanisms underlie the production of artifactual alterations in the membrane of *M. leprae* on the one hand, and of *M. lepraemurium* and *M. avium* on the other. Moreover, our observations show that the extent of damage imparted to the *M. leprae* membrane by aldehyde fixatives is by far more important because the alterations are more serious and more widespread.

The few symmetric membranes found in *in vivo*-grown mycobacteria after fixation with Os-GaFaCa-U probably are membranes altered by degradation by the host's



macrophages since they are present in bacteria without ribosomes and which have lost their PAS-positive components. We have recently shown that change in membrane geometry (with loss of Thiéry-positive molecules) together with ribosome disintegration is an early ultrastructural event in the degradation of mycobacteria by macrophages *in vivo* (8).

In conclusion, the present results show that, in contrast to the cultivable mycobacteria studied, the agent of human leprosy has membranes that are highly sensitive to aldehyde fixation used routinely for electron microscopy. The molecular basis of such a peculiarity remains to be elucidated.

### SUMMARY

In previous reports on the ultrastructure of *Mycobacterium leprae*, we described the occurrence of symmetric membranes in normal-looking bacilli from fresh or frozen samples primarily fixed with aldehydes. In those reports we admitted that such a symmetric profile, which is not found in the other normal mycobacteria, would not represent the structure of the normal membrane of the leprosy bacillus. We, therefore, re-analyzed the ultrastructure of the membrane of *M. leprae*. In the present work the micromorphology of the *M. leprae* membrane was studied by transmission electron-microscopy after the fixation of fresh samples by OsO<sub>4</sub> plus calcium followed by glutaraldehyde plus formaldehyde and calcium followed by uranyl acetate. The study of samples from two patients with lepromatous (LL) leprosy, three armadillos with natural leprosy, and one nude mouse with experimental leprosy showed that normal-looking bacilli present in lead-stained sections had asymmetric membranes with a thickness of  $6.49 \pm 0.36$  nm. These membranes showed periodic acid-Schiff (PAS)-positive components exclusively located in the outer half of the bilayer. We demonstrated that the symmetric profile of the *M. leprae* membrane described in our previous reports corresponds, as admitted in those reports, to an abnormal membrane structure. Such an abnormality was now found to result from the use of primary fixation with aldehydes or of samples stored frozen before fixation. These results indicate that,

although ultrastructurally similar to that of the other mycobacteria, the membrane of *M. leprae* has a peculiar sensitivity to fixation by aldehydes. Such a characteristic, which was not found in *M. lepraemurium*, *M. aurum*, *M. avium*, and *M. tuberculosis* H37Ra, must reflect a unique membrane molecular structure, which is presently unknown.

### RESUMEN

En reportes previos sobre la ultraestructura de *Mycobacterium leprae*, describimos la presencia de membranas simétricas en bacilos de apariencia normal, preparados de muestras frescas o congeladas fijadas con aldehídos. En aquellos reportes admitimos que tales perfiles simétricos, los cuales no se encuentran en otras micobacterias normales, no representaban la estructura de la membrana normal del bacilo de la lepra. En el presente trabajo reanalizamos la ultraestructura de la membrana de *M. leprae* por microscopía electrónica, después de la fijación de muestras frescas con OsO<sub>4</sub> más calcio seguida por glutaraldehído más formaldehído y calcio seguido por acetato de uranilo. El estudio de las muestras de 2 pacientes con lepra lepromatosa (LL), de tres armadillos con lepra natural, y de un ratón desnudo con lepra experimental, mostraron que los bacilos de apariencia normal presentes en las secciones teñidas con plomo, tuvieron membranas asimétricas con un grosor de  $6.49 \pm 0.36$  nm. Estas membranas mostraron componentes positivos al ácido peryódico de Schiff localizados exclusivamente en la mitad exterior de la bicapa. En este trabajo demostramos que el perfil simétrico de la membrana de *M. leprae* descrita en nuestros reportes previos, corresponde a una estructura anormal de la membrana. Ahora se encontró que tal anomalía es el resultado de la fijación primaria con aldehídos o del almacenamiento en congelación de las muestras antes de la fijación. Estos resultados indican que aunque ultraestructuralmente similar a la de otras micobacterias, la membrana de *M. leprae* tiene una peculiar sensibilidad a la fijación por aldehídos. Tal característica, la cual no fue encontrada en *M. lepraemurium*, *M. aurum*, *M. avium*, y *M. tuberculosis* H37Ra, debe reflejar una peculiar estructura molecular de la membrana, todavía desconocida.

### RÉSUMÉ

Dans les rapports précédents concernant l'ultrastructure de *Mycobacterium leprae*, on a décrit l'apparition de membranes symétriques chez des bacilles qui paraissent normaux, provenant d'échantillons frais ou congelés fixés initialement avec des aldéhydes. Dans ces études, nous avons admis que des profils symétriques, non retrouvés chez d'autres mycobactéries normales, ne peuvent représenter la structure de la membrane normale du bacille de la lèpre. Dès lors, on

a procédé à une nouvelle analyse de l'ultrastructure de la membrane de *M. leprae*. Dans cette étude, on a étudié la micromorphologie de membranes de *M. leprae* par microscopie électronique après fixation d'échantillons frais par OsO<sub>4</sub> additionné de calcium, et suivi d'une fixation par la glutaraldehyde additionné de formaldéhyde et de calcium, et ensuite par l'uranyl acetate. L'étude des échantillons provenant de deux malades souffrant de lèpre lépromateuse (LL), de trois tatous atteints de lèpre naturelle, et d'une souris glabre expérimentalement injectée par la lèpre, a montré que les bacilles d'apparence normale qui sont présents dans les sections colorées au plomb présentaient des membranes asymétriques d'une épaisseur de  $6,49 \pm 0,36$  nanomètres. Ces membranes montraient des constituants qui étaient positifs à l'acide périodique (periodic acid-Schiff PAS); ces constituants étaient exclusivement situés dans la moitié extérieure de la double couche de la membrane. On a démontré que le profil symétrique de la membrane de *M. leprae* décrite dans nos rapports précédents correspondait, comme on l'avait dit dans ces rapports, à une structure anormale de la membrane. Cette anomalie, ainsi qu'on l'a observé, ne résultait pas d'une fixation initiale par des aldéhydes, ou du fait que les échantillons avaient été entreposés sous forme congelée avant fixation. Ces résultats indiquent que, malgré qu'elle soit semblable à la membrane des autres mycobactéries d'un point de vue ultrastructural, la membrane de *M. leprae* a une sensibilité particulière à la fixation par les aldéhydes. Une telle caractéristique, qui n'a pas été retrouvée chez *M. lepraemurium*, *M. aurum*, *M. avium*, et *M. tuberculosis* H37Ra, doit refléter une structure moléculaire unique de la membrane, restée inconnue jusqu'à présent.

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