Reevaluation of Envelope Profiles and Cytoplasmic Ultrastructure of Mycobacteria Processed by Conventional Embedding and Freeze-Substitution Protocols

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The cell envelope architectures and cytoplasmic structures of Mycobacterium aurum CIPT 1210005, M. fortuitum, M. phlei 425, and M. thermoressistible ATCC 19527 were compared by conventional embedding and freeze-substitution methods. To ascertain the integrity of cells during each stage of the processing regimes, [1-14C]acetate was incorporated into the mycolic acids of mycobacterial walls, and the extraction of labeled mycolic acids was monitored by liquid scintillation counting. Radiolabeled mycolic acids were extracted by both processing methods; however, freeze-substitution resulted in the extraction of markedly less radiolabel. During conventional processing of cells, most of the radiolabel was extracted during the dehydration stage, whereas postsubstitution washes in acetone yielded the greatest loss of radiolabel during freeze-substitution. Conventional embedding frequently produced cells with condensed fibrous nucleoids and occasional mesosomes. Their cell walls were relatively thick (~25 nm) but lacked substance. Freeze-substituted cells appeared more robust, with well-dispersed nucleoids and ribosomes. The walls of all species were much thinner than those of their conventionally processed counterparts, but these stained well, which was an indication of more wall substance; the fabric of these walls, in particular the plasma membrane, appeared highly condensed and tightly apposed to the peptidoglycan. Some species possessed a thick, irregular outer layer that was readily visualized in the absence of exogenous stabilizing agents by freeze-substitution. Since freeze-substituted mycobacteria retained a greater percentage of mycolic acids in their walls, and probably other labile wall and cytoplasmic constituents, we believe that freeze-substitution provides a more accurate image of structural organization in mycobacteria than that achieved by conventional procedures.

Tuberculosis remains a major health problem for humans, particularly in developing countries, with an estimated 8 million cases and 3 million deaths per year worldwide (7). In the United States, more than 22,000 new cases are being reported annually in particular populations. The epidemiology of tuberculosis has undergone a profound transition in recent years (21). An increase in the incidence of infection due to human immunodeficiency virus has resulted in a rise in the number of both typical and atypical mycobacterial infections. The Mycobacterium avium complex and M. kansasii are the most frequently encountered etiologic agents of atypical mycobacterial infections associated with AIDS (21); however, M. xenopi, M. fortuitum, and M. chelonae have also been reported previously (21).

The molecular and structural compositions of the mycobacterial cell wall must play a significant role in its mechanisms of pathogenicity (18, 22, 23). Moreover, the intricate physical relationships among the diverse macromolecules within the wall appear to be responsible for properties such as drug resistance (4), acid fastness (1), hydrophobic interactions (25), and the immunological reactions of mycobacteria to host environments (28). The role of cell wall components in mycobacterial survival in vivo is poorly defined. Further characterization of the wall combined with accurate insight of the spatial distribution of wall macromolecules is necessary to enhance our understanding of the structural and functional role of the cell wall in host-mycobacterium interactions.

Mycobacterial cell walls are complex, multilayered structures composed of an irregular electron-dense outer layer (OL), an electron-translucent region (ETR), and a peptidoglycan (PG) layer (5). Chemical analyses of the OL in M. avium suggested the presence of polysaccharides, glycoproteins, and glycolipids (23). Similar studies remain to be performed on other species. The ETR is generally believed to be composed of mycolarabinogalactan complexes covalently attached to the PG. Mycobacterial walls contain between 30 and 60% (by weight) lipids, which include true waxes, C-mycoside glycopeptidolipids, phenol glycosides, trehalose-containing lipopolysaccharides, sulfolipids, lipoarabinomannan, and mycolic acids (19). Mycolic acids are β-hydroxy fatty acids substituted at the α position with a moderately long aliphatic chain ranging from C28 to C90. These acids, which are a major constituent of the cell wall and are characteristic of the genus Mycobacterium, esterify terminal arabinose residues of the arabinogalactan polymer to produce mycolyl arabinogalactan (19). This complex is covalently attached by a 1-O-phosphoryl group to the 6 position of muramic acid residues within the PG (18). This is complex wall chemistry which contributes to, and makes it difficult to understand, the wall ultrastructure.

The high lipid content of a mycobacterial cell is particularly susceptible to extraction with organic solvents during conventional embedding, yet, surprisingly, the effects of processing protocols on lipid extraction in mycobacteria have not been previously investigated. Freeze-substitution is a relatively new cryotechnique for electron microscopic examination of biological specimens (12). It combines ultrarapid freezing to arrest physiological cellular processes

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(physical cryofixation) with mild chemical fixation to increase bonding, which stabilizes cell structures during dehy-
dration. The resulting specimens are miscible with plastic resins. Since chemical stabilization is performed below the freezing and recrystallization points of cellular water, arte-
facts associated with conventional methods, such as redis-
tribution and loss of cellular components as well as distor-
tion of cell morphology, are minimized (9, 10). Previous
work on the extraction of lipids from *Acholeplasma laid-
lawii*, a bacterium lacking a cell wall, demonstrated that
freeze-substitution extracted lesser amounts of lipids than
conventional methods (31, 32). More recent studies which
focused on the extraction of radiolabeled wall and cytoplas-
mic components from *Bacillus subtilis* and *Escherichia coli*
during processing for freeze-substitution and conventional
embedding also demonstrated that freeze-substitution yields
superior ultrastructural details of bacterial cells (9, 10).

Our study represents the first attempt to examine myco-
bacterial cells by freeze-substitution and to monitor the
extraction of radiolabeled mycolic acids from the walls of
cells during conventional and freeze-substitution protocols.
We also discuss the importance of preserving lipid structures
for the accurate interpretation of ultrastructural images of
mycobacterial cells.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. fortuitum*,
originally isolated from a bovine case of mastitis, was
supplied by S. Rosendal, University of Guelph. *M. phlei* 425
was obtained from the Department of Microbiology, Univer-
sity of Guelph, culture collection; *M. thermoresistible*
ATCC 19527 and *M. aurum* CIPT 1210005 were type strains.
All strains were grown in brain heart infusion broth at 37°C.
All complex media were from Difco Laboratories (Detroit,
Mich.), and all other chemical reagents were from Fisher
Scientific Co. (Toronto, Ontario, Canada).

Growth of bacterial strains was assessed by optical den-
sity measurements at 600 nm (OD<sub>600</sub>) with a path length of
1 cm with a Pye Unicam 8600 Spectrophotometer (Philips
Electronics, Ltd., Scarborough, Ontario, Canada). Each
culture was grown in 250 ml of broth in 1-liter flasks on a
rotary shaker to its mid-exponential growth phase, which
usually yielded OD<sub>600</sub> measurements ranging from 0.4 to 0.8,
and then harvested by centrifugation (8,000 × g for 10 min).
All bacteria except *M. aurum* grew as clumps of cells in
liquid medium, which made accurate OD measurements
difficult. Consequently, aliquots of clumps were removed
and dispersed with a Potter-Elvejehm tissue homogenizer.
This yielded intact, single-cell suspensions that could be
accurately monitored at OD<sub>600</sub>.

Labeling of mycolic acids with [1-<sup>14</sup>Clacetate. The mycolic
acid fraction was labeled by a modification of the method of
Takayama et al. (29). Radiolabeling of the mycolic acid fraction
was achieved by adding 12.5 μCi of [1-<sup>14</sup>C]acetate (specific activity, 56 mCi/mmol; ICN Biomedicals, Inc.,
Mississauga, Ontario, Canada) to 25 ml of an exponential-
phase culture (OD<sub>600</sub>, 0.3 to 0.5) in brain heart infusion
broth. Preliminary experiments showed that the incorpora-
tion of labeled acetate into the mycolic acid fraction was
rapid and maintained maximum levels for 20 min before
it decreased. Therefore, we routinely incubated cultures for 20
min at 37°C and 180 rpm. Growth was terminated by adding
5 ml of 12% (vol/vol) perchloric acid, and the cells were
harvested by centrifugation (8,000 × g, 10 min) and sapon-
ified in 5 ml of 5% (wt/vol) KOH in 50% (vol/vol) ethanol for
4 h at 85°C. After being saponified, the lipids were acidified
with 1.2 ml of 6 N HCl and extracted with 12 ml of diethyl
ether. The ether extract was evaporated to dryness and
resuspended in petroleum ether. Sample extracts were
added to scintillation vials containing ScintiVerse II (Uni-
versal Liquid Scintillation cocktail; Fisher Scientific, Ltd.)
and analyzed for radioactivity.

Preparation and thin-layer chromatography of mycolic acid
methyl esters. Mycolic acids were extracted and converted to
methyl esters by acid methanolysis as previously described
by Minnikin et al. (20).

The methyl ester extracts were applied to two Silica Gel G
plates (500-μm thickness, 20 by 20 cm; Sigma Chemical Co.,
St. Louis, Mo.). Samples of α-methyl esters from *M. tuberc-
ulosis* H37 Ra were used as standards. Each plate was
developed three times with petroleum ether-diethyl ether
(90:10, vol/vol). The presence of separated components was
revealed by spraying with 0.01% (wt/vol) rhodamine B and
10% (vol/vol) ethanol in 0.25 M sodium phosphate, pH 7.2,
or by exposing the plates to iodine vapor.

When radiolabeled methyl esters were separated by thin-
layer chromatography, their presence was identified with
iodine vapor. One-centimeter bands were scraped directly
into scintillation vials containing 10 ml of toluene in the
presence of Omnifluor (New England Nuclear Corp., Bos-
ton, Mass.), and the radioactivity in samples was measured
with a Tri-Carb 2000 liquid scintillation counter (United
Technologies Packard, Downers Grove, Ill.).

Conventional embedding for electron microscopy. Cells
were harvested by centrifugation (8,000 × g, 10 min) and
washed twice in 100 mM HEPES (N-2-hydroxyethylpiper-
azine-N'-2-ethanesulfonic acid) buffer, pH 6.8 (Research
Organics Inc.). The resulting pellet was resuspended in 4% (vol/vol)
glutaraldehyde (Marivac, Ltd.) in 100 mM HEPES buffer
for 2 h at room temperature. The cells were washed five
times in HEPES buffer, postfixed for 2 h at room
temperature in 2% (wt/vol) aqueous osmium tetroxide,
and then washed again in buffer. After being washed five times
in 50 mM HEPES buffer, cells were suspended in 2% (wt/vol)
aqueous uranyl acetate (Fisher Scientific) for 1 h at room
temperature and then washed five times in distilled water.
The cells were pelleted, enrobod in 2% Noble agar, and cut
to form blocks (3 by 1 mm). Sample blocks were then
dehydrated through a graded acetone series of 25, 50, 70,
and 95% for 15 min each and then washed three times for 15 min
each in 100% acetone. Blocks were infiltrated overnight at
room temperature in 100% aceton–Epon 812 (1:1), embed-
ded in fresh Epon 812 resin, and polymerized at 60°C for 36
h.

Alternatively, blocks were dehydrated through a graded
ethanol series of 25, 50, 70, and 95% for 15 min each and
then washed three times for 15 min each in 100% ethanol, once
for 15 min in ethanol-propylene oxide (1:1), and three times
for 10 min each in 100% propylene oxide (transition stage). After
being infiltrated overnight in Epon 812-propylene oxide
(1:1), blocks were embedded in fresh resin and polymerized.

Freeze-substitution. Freeze-substitution was performed as
described by Graham and Beveridge (9). Cells were
harvested by centrifugation and incubated in 18% (vol/vol)
glycerol (Fisher Scientific) in 50 mM HEPES buffer, pH 6.8,
for 20 min at room temperature. After the cells were pelleted
in an Eppendorf tube, a volume of molten 2% (wt/vol) Noble
agar equal to that of the pellet was added and mixed. This
suspenison was immediately layered onto a sterile cellulose-
ester membrane filter (Gelman Sciences) with a clean glass
microscope slide. Wedge-shaped pieces of this filter were
TABLE 1. Relative distribution of radioactivity in separated components of methyl mycolates from M. aurum CIP 1210005, M. fortuitum, M. phlei 425, and M. thermoresistible ATCC 19527

<table>
<thead>
<tr>
<th>Separated components</th>
<th>% cpm in processing fluids of the following species:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M. aurum</td>
</tr>
<tr>
<td>α-Mycolates</td>
<td>65</td>
</tr>
<tr>
<td>Methoxymycolates</td>
<td>4</td>
</tr>
<tr>
<td>Ketomycolates</td>
<td>31</td>
</tr>
</tbody>
</table>

* Not found in M. fortuitum.

plunge-frozen (pointed end first) in liquid propane at −196°C. Frozen samples were then transferred to glass vials containing frozen substitution media and molecular sieves (sodium alumino silicate; pore size, 0.4 nm; Sigma Chemical Co.) maintained at −196°C and gently pressed onto the surface to ensure that the cells were in direct contact with the frozen medium. Substitution media were prepared fresh prior to being used and consisted of 2% (wt/vol) osmium tetroxide and 2% (wt/vol) uranyl acetate in anhydrous acetone. In order to minimize drying of the cell suspension, the time that elapsed between the layering of the sample on the membrane and the transfer of plunge-frozen cells into substitution media rarely exceeded 2 min. After transfer, the vials were sealed and the specimens were cryosubstituted at −80°C for 72 h. After substitution, the vials were removed and allowed to reach room temperature. Samples were washed six times for 15 min each in anhydrous acetone and then infiltrated overnight at room temperature in 100% acetone–Epon 812 (1:1). Samples were embedded in fresh Epon 812 and polymerized at 60°C for 36 h.

Electron microscopy. Bacteria prepared by conventional embedding and freeze-substitution were thin sectioned on a Reichert-Jung Ultracut ultramicrotome and mounted on Formvar carbon-coated copper grids. Sections were poststained first in 2% (wt/vol) aqueous uranyl acetate and then in lead citrate. Electron microscopy was performed with a Philips EM 300 operating at 60 kV under standard conditions with a liquid-nitrogen cold trap in place.

Statistical analyses. A factorial analysis of variance in a randomized design (8) was used to analyze sample means in order to determine the effects of processing treatments on radiolabel extraction within species, radiolabel extraction between species, and the interaction of treatment and species.

RESULTS

Extraction of radiolabeled mycolic acids from conventionally prepared cells. To measure the extent of leaching of mycolic acids from mycobacterial walls during processing for electron microscopy, we labeled mycolic acids with [1-14C]acetate. Separation of radiolabeled methyl mycolates by thin-layer chromatography revealed that uptake of the radioisotope was rapid and that maximum incorporation of radioactivity was achieved in the mycolic acid fractions. These findings are consistent with those of previous studies on the uptake of labeled metabolites into mycolic acid fractions (16, 17, 29). Table 1 shows the relative distribution of radioactivity in the mycolic acids separated by our system. The majority of radiolabel was found in the α-mycolate fraction, and relatively little was associated with the methoxymycolate component, when it was present. These data suggest that the amount of radioactivity incorporated could reflect the relative abundance of mycolic acids in a given species.

Four mycobacterial species were labeled in the mycolic acid fraction with [1-14C]acetate. Figure 1 shows the total percent counts per minute detected in processing fluids when bacteria were prepared by conventional methods with acetone or ethanol as the dehydrating agent. Less than 4% of the total of radiolabeled mycolic acids (in counts per minute) was...
detected in processing fluids following glutaraldehyde, osmium, or uranyl acetate fixation. In contrast, the majority of radiolabel was extracted from cells during the dehydration stage. In particular, significantly (P < 0.001) greater amounts of radiolabel were extracted from cells dehydrated with acetone than from cells dehydrated with ethanol. The degree of leaching was inversely proportional to the concentration of acetone; however, no correlation between ethanol concentration and the degree of radiolabel solubilization was found. With the exception of M. fortuitum and M. thermoresistible, relatively low levels of radiolabel were detected during plastic infiltration following dehydration in acetone (Fig. 1). The transition phase through propylene oxide induced some leaching of radiolabel, while lower levels of radiolabel were found in the infiltration media. No significant difference in radiolabel extraction was observed between species, regardless of the dehydrating solvent (Table 2).

**Extraction of radiolabeled mycolic acids from freeze-substituted cells.** Some radiolabel was extracted during the substitution and infiltration phases of freeze-substitution, but the majority of label was lost during the acetone washes (Fig. 2). In particular, the first three acetone washes of the series solubilized the most labeled material, whereas successive washes extracted less label. Similar levels of mycolic acids were leached from all four species during freeze-substitution, but profoundly less label was extracted than during conventional embedding (i.e., from 11% difference between ethanol conventional regimen and freeze-substitution of M. aurum to 41% difference between acetone conventional regimen and freeze-substitution of M. fortuitum) (Table 2).

**Envelope profiles and cytoplasmic ultrastructure of conventionally processed bacteria.** Electron microscopic examination of four mycobacterial species processed by a conventional method and dehydrated with acetone or ethanol revealed similar cell wall architecture but markedly distinct cytoplasmic features (Fig. 3 to 6). Interestingly, we consistently found that acetone-dehydrated cells revealed greater staining intensity (i.e., the cells were darker) and poorer overall contrast than their ethanol-dehydrated counterparts. Acetone affected the staining of these sections, and this sometimes made it difficult to discern cell wall layers and cytoplasmic organelles.

All species possessed an electron-dense OL, approximately 5 nm in width. In particular, the OLs of M. phlei (Fig. 4) and M. thermoresistible (Fig. 5) were most conspicuous, whereas the OLs of M. fortuitum and M. aurum cells were very thin and poorly discernible, particularly when dehydration was performed with ethanol (Fig. 3) and acetone (Fig. 6), respectively. Profiles also revealed an ETR, 5 to 10 nm in width, overlying a heavily stained PG band, 5.6 to 6.2 nm in width. All plasma membranes were typically wavy bilayered structures of uniform width (2.5 nm) and staining intensity (Fig. 12 and 13).

The cytoplasm of acetone-dehydrated cells was often perforated with electron-translucent voids and contained ribosomes that were difficult to distinguish from the cytoplasmic contents. The nucleoid was condensed centrally within the cytoplasm and consisted of intertwined DNA strands. The cytoplasm of ethanol-dehydrated cells possessed a uniform appearance with heavily stained ribosomes.

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**TABLE 2.** Total percent counts per minute detected in processing fluids during conventional embedding and freeze-substitution of *M. aurum* CIPT 1210005, *M. fortuitum*, *M. phlei* 425, and *M. thermoresistible* ATCC 19527

<table>
<thead>
<tr>
<th>Processing method</th>
<th>% cpm in processing fluids of the following species:</th>
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<tbody>
<tr>
<td></td>
<td>M. aurum</td>
</tr>
<tr>
<td>Conventional embedding</td>
<td></td>
</tr>
<tr>
<td>Dehydration with acetone</td>
<td>55.0 ± 3.1</td>
</tr>
<tr>
<td>Dehydration with ethanol</td>
<td>43.75 ± 2.4</td>
</tr>
<tr>
<td>Freeze-substitution</td>
<td>32.77 ± 2.4</td>
</tr>
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</table>

* All values represent the means of two independent experiments, each containing two sample replicates.

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**FIG. 2.** Percent counts per minute detected at different stages during freeze-substitution of *M. aurum* CIPT 1210005, *M. fortuitum*, *M. phlei* 425, and *M. thermoresistible* ATCC 19527. Open bars, cryoprotectant (C); stippled bars, substitution medium (S); solid bars, acetone washes (A); hatched bars, infiltration stage (I).
FIG. 3-6. Thin sections of mycobacteria prepared by conventional embedding with acetone or ethanol as the dehydrating agent. All cells dehydrated with ethanol typically revealed distinct ribosomes (open arrowheads) and a nucleoid region which was confined to the cell center and consisted of highly coiled DNA strands (Fig. 3 and 4). The cytoplasm of cells dehydrated with acetone was often perforated with electron-translucent voids and a centrally condensed nucleoid containing randomly distributed DNA fibers. Note the presence of the OL (solid arrowheads) external to the heavily stained PG layer (those of M. aurum and M. fortuitum are difficult to see except for those regions in good cross-section). Bar = 100 nm.

The strains shown in Fig. 3 to 6 are representative of all strains examined.

FIG. 3. M. fortuitum with ethanol as the dehydrating agent.
FIG. 4. M. phlei 425 with ethanol as the dehydrating agent.
FIG. 5. M. thermoacetica ATCC 19527 with acetone as the dehydrating agent.
FIG. 6. M. aurum CIPT 1210005 with acetone as the dehydrating agent.
FIG. 7-10. Thin sections of mycobacteria prepared by freeze-substitution. Note that fine DNA fibrils are distributed throughout the cytoplasm. Arrowheads indicate OLs. Bar = 100 nm.
FIG. 7. *M. phlei* 425.
FIG. 8. *M. thermoresistible* ATCC 19527.
FIG. 9. *M. aurum* CIPT 1210005.
FIG. 10. *M. fortuitum*.
that could be seen clearly in sections less contrasted than those in Fig. 3 and 4. The nucleoid was even more condensed, and it was confined to the cell center and composed of more highly coiled bundles of DNA strands (compare Fig. 3 and 5).

Envelopes profiles and cell morphologies of freeze-substituted bacteria. Freeze-substituted bacteria possessed thinner walls (23.5 ± 2.7 nm) than their conventionally embedded counterparts (acetone dehydrated, 25.9 ± 2.6 nm; ethanol dehydrated, 25.8 ± 2.7 nm), but the measurable difference between walls was not significant. Compared with conventionally processed cells, freeze-substituted bacteria also revealed a multilayered cell envelope in which the plasma membrane was tightly associated with the PG layer (Fig. 12 to 15). Freeze-substituted cells of *M. phlei* (Fig. 7) and *M. thermoresistible* (Fig. 8) possessed thick, irregular, heavily stained OLs, 5.6 to 10.6 nm in width, that were readily visualized prior to poststaining (Fig. 11). Our ability to visualize the OL through the contrast imparted only by the uranyl ions and osmium tetroxide within the substitution medium attests to the exquisite ultrastructural details that can be obtained by freeze-substitution (Fig. 11). The OL would not be readily visualized in unstained thin sections of conventionally processed cells. The OLs of *M. aurum* (Fig. 9) and *M. fortuitum* (Fig. 10) were frequently difficult to visualize after poststaining, except for those areas in precise cross-section alignment.

The PG was present as an electron-dense band, 5.6 to 6.2 nm in width. All plasma membranes were smooth, asymmetrically stained structures (the outer face possessed more heavy-metal stain than the inner face) of uniform width (2 nm) underlying the PG (Fig. 7 to 10, 14, and 15). The cytoplasm was characterized by widely separated, robust ribosomes intermeshed with a dispersed nucleoid typical of freeze-substituted bacteria described previously by other workers (14).

**DISCUSSION**

Much of the information concerning mycobacterial wall ultrastructure has been derived from freeze-fracture studies (1), negative staining (15), and thin sections of conventionally prepared bacteria (5, 24). It has been difficult to reconcile the ultrastuctural images derived from these techniques, and these images have not always correlated properly with chemical analyses of purified isolated wall preparations. The topographical relationships among wall constituents have also been difficult to visualize accurately by conventional fixation and embedding methods. These discrepancies may be due to the intricate nature of the cell wall and the potential of electron microscopic techniques to yield artifacts (13). Despite these shortcomings, several models have been previously proposed to describe mycobacterial wall architecture (1, 18, 19, 23). Through negative staining and freeze-fracture studies, Barksdale and Kim (1) elaborated on an earlier model of Imaeda et al. (15) to propose a four-layer wall comprising a surface glycolipid or glycopetidolipid layer (L1) overlying two layers containing lipopolysaccharide complexes (L2 and L3) and a PG layer (L4). A speculative model based on lipid biochemistry and their biophysical organization in eubacterial membranes attempted to accommodate the interactions between heterogeneous lipid polymers by proposing the formation of an asymmetrical lipid matrix (19). In this chemical model, mycolic acids would be packed with their hydrocarbon chains parallel to form a monolayer permeability barrier. Interactions of other complex lipids with mycolic acids would then contribute to the integrity of the mycolic acid lipid matrix. Other attractive proposals based on this model have been recently discussed (18, 22); however, there is currently little direct evidence to support these models.

Radiolabeled acetate is a valuable metabolic marker and has been shown to be incorporated into several types of
mycolic acid in *M. phlei* (16), *M. aurum* (17), and *M. tuberculosis* (29). However, labeled acetate is probably also incorporated into other cell wall lipid polymers in mycobacteria. We did not identify these structures but instead demonstrated that mycolic acids, which constitute the major wall lipid in mycobacteria, incorporated most of the radiolabel in our four species. On the basis of this result, we believe that radiolabeled lipids would enable us to monitor directly the extent of cell wall damage incurred during conventional embedding and freeze-substitution of mycobacteria. Negligible amounts of labeled mycolic acids were lost during fixation with glutaraldehyde, osmium, or uranyl acetate. However, the lack of mycolic acids in processing fluids does not preclude their redistribution within the cell wall. The dehydration stage resulted in the greatest extraction of mycolic acids from conventionally processed cells. In particular, the levels of mycolic acids leached during dehydration with acetone were greater than those leached during dehydration with ethanol. Similarly, during freeze-substitution, the majority of radiolabel was detected in the acetone washes. The dehydration stage of conventional embedding tends to distort the mycobacterial envelope through the removal of water from polar wall polymers such as PG and arabinogalactan (6). If a proportion of these structures is solubilized and extracted during processing, it is possible that mycolic acids, which are covalently bound to the PG-arabinogalactan complex, will also be removed. No correlation between a total loss of mycolic acids and the different species processed by conventional methods was found, i.e., all species lost comparable amounts of label. During freeze-substitution, all species lost similar amounts of labeled material but markedly less label than cells processed by conventional embedding (Table 2).

The magnitude of mycolic acids extracted from the cell walls of mycobacteria processed by both techniques would imply modifications in wall architecture among different species. Indeed, electron microscopic examination revealed marked differences in the cell envelope profiles and the cytoplasmic morphologies of cells prepared by both techniques. Although as much as 34% of the mycolic acids (by weight) were extracted from mycobacterial walls during freeze-substitution, our electron microscopic profiles of processed cells revealed well-frozen, turgid bacteria consisting of a dispersed nucleoid and multilayered envelopes. In accordance with the findings of Graham and Beveridge (9), we believe that freeze-damage of mycobacterial cells could explain the major loss of mycolic acids during processing. Freeze-damage must occur to those cells which are deeply embedded (i.e., ∼10 μm below the ice surface) within the film of bacteria to be frozen. These cells would be more susceptible to leakage during subsequent chemical treatments. Therefore, only those cells that undergo rapid freezing near the top of the film remain intact and are adequately preserved to resist subsequent processing (9).
Accurate visualization of mycobacterial surfaces has been hampered by a lack of alternative methods for the preservation of surface structures in their native states. Earlier work using ruthenium red demonstrated the presence of an OL in several mycobacterial species (24). This layer was not visualized by poststaining with lead citrate, except for an occasional thin heavy-metal deposit that demarcated the interface of the OL and ETR (24). We observed an OL in each species processed by conventional embedding after being poststained with lead citrate and uranyl acetate. The composition of the OL is poorly defined in the majority of species, but in M. avium, it is reported to be rich in polysaccharides and glycoproteins (23). It is possible that these surface polymers are hydrated and would suffer some degree of collapse, as well as extraction, during the dehydration stages of conventional processing. The addition of ruthenium red may stabilize these polymers, thereby minimizing their collapse during dehydration and retaining their structures. However, much of the resulting ultrastructure and mass of these stabilized structures has been shown to be attributed to the presence of the stabilizing agent (2).

Freeze-substitution has proved to be an excellent cryotechnique for the preservation of delicate surface structures such as capsular polysaccharides and S layers (11). Our freeze-substituted profiles revealed thick, irregular, heavily stained OLs on the surfaces of M. phlei and M. thermore sistible that were the only readily visualized structures prior to poststaining (Fig. 11). These findings suggest that the OLs of some mycobacterial species can be readily visualized by freeze-substitution in the absence of exogenous stabilizing agents. The OLs of M. fortuitum and M. aurum cells processed by both techniques were very thin and difficult to visualize. We have examined more than 100 thin sections of cells of each strain to demonstrate the reproducibility of this structural phenomenon. We believe that a relatively thin OL may be a characteristic feature of these strains. Further studies are necessary to determine whether other M. fortuitum and M. aurum strains share this structural feature and to determine the role that growth conditions, growth phase, or nutritional factors may play in influencing mycobacterial surface composition and structure. Our results indicate that variation in OL thickness exists between the species examined in this study.

During conventional embedding, the cell suffers excessive losses of wall and cytoplasmic components (9, 10, 13, 30, 31, 32), which in turn disrupt the organization of the remaining macromolecules. We examined a single major wall constituent in this study, but it has been demonstrated previously that other radiolabeled wall polymers and cytoplasmic material are extracted from B. subtilis and E. coli during conventional embedding and freeze-substitution (9, 10). Therefore, it would seem unlikely that mycolic acids were the sole components extracted during processing. It is possible that during conventional processing, the bonding forces of the cell envelope macromolecules are so disrupted that the fabric of the cell envelope has separated and expanded to an unnatural thickness. This would allow the leaching away of many cytoplasmic substances. Indeed, a variable space between the PG layer and the wavy plasma membranes was apparent in most conventionally prepared cells, whereas a thin, uniform spatial region was bordered by thin, smooth plasma membranes and a PG band in freeze-substituted cells (Fig. 12 to 15). Paradoxically, the ETR and OL were thicker in profiles of freeze-substituted cells. The ETR is believed to be the site in which mycolic acids, glycolipids, glycopeptidolipids, and possibly other lipid polymers are packed in an ordered configuration to form an enveloping permeability barrier (19). Since less mycolic acid was leaked from the walls of freeze-substituted cells than from the walls of conventionally processed cells, we propose that a thicker ETR reflects better preservation of labile wall constituents, particularly lipid polymers, by freeze-substitution.

Our proposal received further support from our demonstrating for the first time freeze-substitution of asymmetrically stained plasma membranes. Asymmetrical distribution of plasma membrane phospholipids and proteins in mycobacteria has been previously demonstrated by conventional embedding methods (26, 27); however, the preservation of membrane asymmetry was dependent upon the type of chemical fixative and embedding resin employed in processing, as well as the stain used for contrasting the membranes (26, 27). Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, and phosphatidylinositol are the common structural amphipathic lipids in mycobacterial membranes (3) that would offer available hydroxyl, carboxyl, and phosphoryl groups for reactions with osmium and uranyl ions. The asymmetry of staining in the outer leaflet reflects greater deposition of osmium and uranyl ions onto the polar groups of phospholipids or proteins. Accordingly, the higher contrast in the outer face could be attributed to a natural predominance of phospholipids or proteins in the outer face. The absence of such staining in our conventionally processed cells may be due to the loss or the redistribution of phospholipids or proteins throughout both faces of the plasma membranes induced by inadequate lipid-protein stabilization. Our findings are consistent with those of earlier studies which showed that plasma membrane lipids of the wall-less bacterium A. laidlawii were better preserved by freeze-substitution than by conventional embedding (31, 32).

The cytoplasmic ultrastructure revealed marked differences in cells processed by both techniques. Differences in cells dehydrated with acetone or ethanol during conventional embedding were also apparent. The deleterious effects of dehydration with acetone were strikingly apparent upon examination of the cell cytoplasm. Ultrastructural images of acetone-dehydrated cells revealed a perforated cytoplasm, with indistinct ribosomes and a condensed nucleoid consisting of loosely organized DNA strands. In contrast, the cytoplasm of ethanol-dehydrated cells had a uniform appearance that revealed heavily stained ribosomes and a nucleoid that was more compact and confined to the cell center and that comprised bundles of DNA fibrils.

The gross morphologies of nucleoids and the fine structural organization of DNA have been previously shown to be altered by various treatments, including aldehyde or osmium fixation during conventional embedding (14). Since we maintained the same fixation protocol (glutaraldehyde followed by osmium tetroxide and uranyl acetate) prior to dehydration in either acetone or ethanol, we suggest that the type of dehydration solvent is an additional factor that influences nucleoid morphology in conventionally prepared mycobacteria. The cytoplasm of freeze-substituted mycobacteria revealed an extensive ribosome-free region that restricted ribosomes to the periphery of this area and contained fine DNA fibrils distributed throughout. Our results are in agreement with the findings of Hobot et al. (14), who previously reported the presence of ribosome-free regions in freeze-substituted cells of E. coli and B. subtilis. Their evidence and our data support the notion that rearrangement of DNA and cytoplasmic material is minimized during freeze-substitution. Consequently, the different nucleoid shapes observed after chemical fixation appear to be artificial.
Our interpretation of mycobacterial wall architecture, as revealed by freeze-substitution, could help to explain the apparent low permeability of mycobacteria. Condensation of the wall fabric would mean less penetration of antibiotics and dyes through the cell envelope. We feel that freeze-substitution could be a valuable technique to help resolve some of the ultrastructural and chemical conflicts that currently prevail in our understanding of the relationships between heterogeneous macromolecules in mycobacterial envelopes, especially at a time when there is an increasing awareness of the role of cell surfaces in mycobacterial pathogenicity.

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