The Walls of *Mycobacterium lepraemurium*: Chemistry and Ultrastructure

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SUMMARY

Methods are described for isolating the walls of the intracellular parasite *Mycobacterium lepraemurium* in good yields. The walls were treated with solvents and reagents and their effects were followed by chemical analyses and electron microscopy. The walls resemble those of other mycobacteria, consisting of mucopolypeptide, arabinogalactan and ester-linked lipid. The lipid forms the outer, electron-transparent layer of the wall. The information available so far offers no explanation of the bacterium's outstanding resistance to destruction by phagocytic cells.

INTRODUCTION

*Mycobacterium lepraemurium* is an obligate parasite of rats and mice. It is of interest because of its extraordinarily successful adaptation to existence inside phagocytic cells of the host; it multiplies without appearing to damage the cell and without itself being affected by the bactericidal or bacteriolytic mechanisms of the cell. This suggests that the outer layers of the bacterium may show unusual features. Cummins, Atfield, Rees & Valentine (1967) published information on the composition of its walls, but used rather poorly characterized preparations. Kanetsuna, Imaeda & San Blas (1968) analysed a single wall preparation from the Hawaii strain of the organism, prepared by a standard technique devised for walls from other mycobacteria (Kanetsuna, 1968). In the present work I have used the electron microscope and chemical analysis to investigate the effects of purification and chemical 'dissection' of the walls.

METHODS

Experimental infection. Albino mice (Parkes' strain) were infected intravenously with a suspension of *Mycobacterium lepraemurium* strain DOUGLAS (Balfour-Jones, 1937) obtained from infected mice (Rees, Valentine & Wong, 1960). In 4 to 5 months, when the animals were visibly sick, they were killed with coal-gas or carbon dioxide gas, and their livers and spleens were removed.

Suspension of bacteria – method 1 (modified from Rees, Valentine & Wong, 1960). The livers and spleens were homogenized in a glass tissue-grinder in 0.5 M-tris-HCl buffer, pH 7.4, containing 0.05 M-CaCl₂ (10 ml. per animal). The suspension was centrifuged at 300 g for 5 min. and the supernatant material digested with pronase (40 µg./ml.) for 16 h. at 37°. Bacteria were collected from the digest by centrifuging (10,000 g for 10 min.), washed several times with 0.1 M-tris-HCl buffer pH 7.2 containing 0.05% Tween 80 (tris-Tween), and suspended in the same buffer.

Suspension of bacteria – method 2. Livers and spleens were chopped with scissors and homogenized in a Potter–Elvehjem homogenizer with a Teflon pestle in chilled 0.3 M-sucrose
buffered to pH 7.2 with 2.5 mM-potassium EDTA (SE, 15 ml./animal). The homogenate was centrifuged at 500 g for 5 min., the sediment resuspended in SE, centrifuged under the same conditions, and the combined supernatants were centrifuged at 10,000 g for 10 min. The sediment was washed twice with chilled SE, the fluffy superficial layer of mitochondria poured off, and the sediment suspended in Triton X-100 (1% in SE) and kept at room temperature for 15 min. The purified bacteria were collected by centrifuging at 10,000 g for 10 min. If any visible trace of mitochondria or erythrocytes remained the treatment with Triton was repeated. The bacteria were washed with SE, then with tris-Tween and finally suspended in tris-Tween (about 40 ml./5 mice originally used).

Crude walls. The bacteria were broken in a chilled French press (Aminco, Silver Spring, Maryland, U.S.A.; Milner, Lawrence & French, 1950), first at 9020 p.s.i. to fragment clumps of bacteria and then twice at 22,600 p.s.i. Small amounts (about 1 mg.) of ribonuclease and deoxyribonuclease, and benzyl penicillin (60 mg./50 ml.), were added, and the suspension was left at 4° for 16 h. then for a further 24 h. at 4° with trypsin (100 μg./ml.). The walls and unbroken bacteria were collected by centrifuging (12,000 g, 10 min.), washed with tris-Tween and suspended in sucrose (15% w/v in tris-Tween). The suspension was layered on to a discontinuous sucrose density gradient consisting of layers of sucrose (20, 25 and 30% (w/v) in tris-Tween). The gradient was centrifuged in a swing-out rotor for 2 to 3 h. at 3000 g, and the layer containing the walls removed. The crude walls were washed several times in tris-Tween, then several times in 0.05% Tween 80, once in water and freeze-dried.

'Dissection' of walls. Crude walls were suspended in 2% sodium dodecyl sulphate for 24 h. at room temperature, washed with water, m-NaCl, several times with water, and freeze-dried to yield 'membrane-free' walls. Lipids were extracted with chloroform–methanol (1:1 by vol.) for 3 to 4 days at room temperature. When fractionation of the lipids was desired, walls were extracted with acetone for 24 h., ethanol–ether (1:1 by vol.) for 3 to 4 days and then with chloroform–methanol as above.

'Bound' lipids were removed by treating the walls with KOH (0.5% in ethanol) for 4 days at 37°. The solid residue was washed with ethanol, extracted with chloroform–methanol (2:1 by vol.) and dried to yield 'lipid-free' walls. The 'bound' lipid was recovered from the combined ethanolic KOH and chloroform–methanol extracts by neutralizing with HCl and partitioning between water and chloroform.

Carbohydrate was removed from the lipid-free walls by treating them with 0.1 N-H₂SO₄ for 3 days at 37°. The solid residue was washed with water and freeze-dried. The soluble carbohydrate was recovered by drying the solution after neutralizing with BaCO₃ and filtering.

Lysozyme treatment. Lipid-free, acid-treated walls (3 mg. in 4.5 ml. of 0.067 m-phosphate, pH 7) were treated with 0.5 ml. of egg-white lysozyme (1 mg./ml.) and incubated at 37° for 3 days. The insoluble material was washed with m-NaCl and with water. N-Acetylhexosamine was estimated in the supernatant by the method of Reissig, Strominger & Leloir (1955).

Phosphodiesterase treatment. An attempt was made to remove the carbohydrate from lipid-free walls with phosphodiesterase: 11.3 mg. of walls were suspended in 5 ml. of 0.1 M-tris-HCl buffer, pH 8.5, with 0.5 units of phosphodiesterase (Crotalus adamanteus phosphodiesterase, type II, Sigma (London) Chemical Co. Ltd) and incubated at 37°.

Preparation of dinitrophenyl-walls. Lipid- and carbohydrate-free walls (5.2 mg.) were suspended in 4 ml. of 1% sodium tetraborate; 1.2 ml. of ethanol containing 12 μl. of dinitrofluorobenzene were added and the mixture was kept at 50° for 3½ h. The DNP-walls were collected, washed with water, dried and hydrolysed with 4 N-HCl at 105° for 16 h. in
Walls of Mycobacterium lepraemurium

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a sealed tube in vacuo. Water-soluble and ether-soluble DNP-amino acids were chromato-
graphed on cellulose thin-layer plates, using as solvents butanol–acetic acid–water (4:1:5 by
vol.) and tert-amyl alcohol saturated with 0.1 M-potassium phthalate buffer, pH 6. Diamino-
pimelic acid was recovered from the separated mono-DNP-amino acid by hydrolysis in
saturated aqueous Ba(OH)_2 for 1 h. at 100° (Fraenkel-Conrat, Harris & Levy, 1955).

Phenol extraction of walls. A sample of walls, extracted with chloroform–methanol (1:1 by
vol.), was treated with phenol (90% in water) at 70° for 24 h. The phenol-insoluble material
was removed by centrifuging at room temperature and the phenol extract dialysed against
distilled water at room temperature. The water-insoluble material precipitated by removal
of the phenol was collected, and the phenol-soluble water-soluble material obtained by
evaporating the supernatant.

Analytical methods. The bound lipid fraction was examined by thin-layer chromatography
on silica-gel, using a two-solvent system: ether–petroleum spirit (b. p. 40° to 60°)–acetic acid
(65:35:0.5 by vol.), followed by ether–petroleum spirit (6:94 by vol.). Compounds were
detected by spraying with conc. H_2SO_4–water (1:1 by vol.) and heating.

Carbohydrates were estimated before or after hydrolysis by the phenol-H_2SO_4 method of
Dubois, Gilles, Hamilton, Rebers & Smith (1956), using galactose as a standard. Mono-
saccharides were obtained by hydrolysis in 2N-H_2SO_4 for 3 h. at 105°. The acid was removed
with BaCO_3, and the sugars examined by cellulose thin-layer chromatography using the
following solvents: formic acid–ethyl methyl ketone–tert-butanol–water (15:30:40:15 by
vol.), and by gas–liquid chromatography of trimethylsilyl ethers. Galactose was estimated
specifically with ‘Galactostat’ reagent (Worthington Biochemical Corp., Freehold, New
Jersey, U.S.A.). Methyl pentose was estimated by the method of Dische (1962), using a 10 min.
heating period and rhamnose as a standard. Hexosamines and muramic acid were
estimated after hydrolysis with 4N-HCl at 100° for 4 h., with an automatic amino acid analyser
or by the method of Blix (1948). Using the latter method, measurement of E_530 and E_505
after 24 h. allowed glucosamine and muramic acid to be calculated separately (Crumpton,
1959). Hexosamines and amino acids were measured with the amino acid analyser after
hydrolysis with 4N-HCl at 105° for 16 h. Acid was removed from hydrolysates in vacuo
over NaOH.

Phosphorus was estimated by the method of Chen, Toribara & Warner (1956), and amino
nitrogen with ninhydrin after acid hydrolysis (Rosen, 1957).

Electron microscopy. Walls and fractions were fixed in OsO_4 (1% in pH 6 Kellenberger
buffer; Kellenberger, Ryter & Séchaud, 1958), post-fixed in uranyl acetate (1% in the same
buffer), embedded in agar, dehydrated with acetone, embedded in Araldite or TAAB
embedding resin (TAAB Laboratories, Reading, Berkshire) and sectioned with a Porter-
Blum MT I microtome. For some samples, 2.5% glutaraldehyde was used before OsO_4. In
some experiments ruthenium red was added to the fixative (Luft, 1966). Carbohydrates were
detected by treating unfixed walls with silver methenamine (de Martino & Zamboni, 1967)
after oxidation with chromic acid or periodic acid.

Suspensions of walls were examined by negative staining with 2% sodium silicotungstate
or 1% uranyl acetate.

RESULTS

Crude walls. Walls from Mycobacterium lepraemurium broken in the French press re-
ained suspended in 25% sucrose in the sucrose density gradients. The composition of the
washed walls is shown in Table 1. Allowing for 25% of bound lipid (found in the walls after
SDS treatment) and assuming a mean molecular weight of 100 for the nitrogenous material, over 90% of the dry weight was accounted for. Gas chromatography of trimethylsilyl derivatives indicated that the crude walls contained arabinose, galactose, mannose and glucose. Typically 50 mg. of walls were obtained from livers and spleens of 5 mice.

Table 1. Composition of crude walls of Mycobacterium lepraemurium
isolated from sucrose density gradients

<table>
<thead>
<tr>
<th>Composition</th>
<th>Percentage of Dry Weight ± Standard Deviation</th>
<th>Number of Batches in Brackets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone-soluble lipid</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Ethanol-ether soluble lipid</td>
<td>22 ± 3 (4)</td>
<td></td>
</tr>
<tr>
<td>Total 'unbound' lipid</td>
<td>31 ± 9 (6)</td>
<td></td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.31 ± 0.15 (3)</td>
<td></td>
</tr>
<tr>
<td>Nitrogen</td>
<td>2.5 ± 0.3 (3)</td>
<td></td>
</tr>
<tr>
<td>Total neutral sugar*</td>
<td>18.9 ± 6.1 (6)</td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>6.5 ± 0.8 (4)</td>
<td></td>
</tr>
<tr>
<td>Methyl pentose</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>Total amino-sugars*</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>Galactosamine*</td>
<td>1.6</td>
<td></td>
</tr>
</tbody>
</table>

*a Expressed as galactose;  b expressed as rhamnose;  c expressed as glucosamine;  d estimated as difference between Galactostat assays before and after passing hydrolysate through short column of Dowex 1.

In the electron microscope the material was seen to consist of wall fragments ranging from nearly intact bacteria opened at one end down to thirds or quarters of the whole (Fig. 1, 2, 3). The broken ends were characteristically curled inwards, as though the outer layers of the wall were under compression (Fig. 4). The wall fragments mostly enclosed membranous or particulate material (Fig. 1, 3, 5), including recognizable mesosomes (Fig. 5). Structurally the wall was of two layers, seen in sections, an inner electron-dense layer about 5 nm. thick and an outer electron-transparent layer about 10 nm. across. The outer layer was commonly demarcated by a fringe of darker material, and its presence could also be inferred from the appearance of packed walls (Fig. 6).

Treatment with 2% sodium dodecyl sulphate (SDS), or with 0.1% sodium deoxycholate, either in water or in 0.1 N-NaOH, removed material from inside the walls (Fig. 6–8). Removal was, however, incomplete if the ‘unbound’ lipids had first been removed with neutral organic solvents. Of the detergents SDS seemed the more satisfactory, and removed 27% ± 9 S.D. (9 samples) of material from the walls. This included, in one batch, 5% of the neutral sugars excluding the galactose. Apart from the contents, the detergents appeared also to remove material from the surface of the walls, which thereafter became clumped instead of forming a smooth suspension.

Removal of lipids. Extractable lipids, that is lipids removable by neutral organic solvents, could be removed from SDS-treated walls with chloroform–methanol (1:1 by vol.). The

Crude walls of Mycobacterium lepraemurium.

Fig. 1. Sectioned walls showing membranous residues inside. Fixed with OsO₄ and post-fixed with uranyl acetate. Section stained with uranyl acetate.

Fig. 2. Negatively stained walls, showing type of breakage and fibrous structures on surface. Stained with sodium silicotungstate.

Fig. 3. Negatively stained wall showing single incomplete break and membranous (mesosomal?) material adhering. Stained with sodium silicotungstate.

Fig. 4 and 5. Sectioned walls, showing two-layered structure. A remaining mesosome is visible in Fig. 5. Fixed with OsO₄ and post-fixed with uranyl acetate. Section stained with uranyl acetate.
Walls of *Mycobacterium lepraemurium*
Treated walls of *Mycobacterium leprae*. Scale-bars indicate 0.1 μm.

Fig. 6, 7 and 8. Walls extracted with sodium dodecyl sulphate. Arrows indicate electron-transparent layer between adjacent walls. Fixed in glutaraldehyde and OsO₄ (OsO₄ only for Fig. 7) and post-fixed with uranyl acetate. Sections stained with lead citrate.

Fig. 9. Walls extracted with chloroform–methanol, then 0.5% KOH in ethanol. Fixed in OsO₄ and post-fixed with uranyl acetate. Section stained with uranyl acetate.

Fig. 10. Material as Fig. 9, detail.

Fig. 11. Walls extracted with chloroform–methanol, 0.5% KOH in ethanol and finally again with chloroform–methanol. Arrows show absence of clear layer between adjacent walls. Fixed with OsO₄ containing ruthenium red. Section unstained.

Fig. 12. Walls extracted as for Fig. 11. Treated with silver–methenamine. Section stained with uranyl acetate.
residue was extremely hydrophobic, and it was difficult to ensure completeness of the various acid hydrolyses. Subsequent treatment to remove the bound lipids yielded an easily suspended residue. The composition of SDS-treated walls before and after removal of extractable and bound lipids and of deoxycholate-treated walls is shown in Table 2. The lipid-free walls comprised about 60% of the weight of the walls freed from extractable lipids.

The lipid-free walls contained alanine, glutamic acid, diaminopimelic acid, glucosamine and muramic acid in the ratios 1.6:0.9:1.0:0.8:0.5. Traces of several other amino acids were present, of which the most abundant was glycine, in a molar ratio of 0.1. Total amino acid and hexosamine content, corrected for presumed losses of glucosamine and muramic acid in the hydrolyses, was 32% and 86% of this material has been identified as polysaccharide and mucopeptide.

Thin-layer chromatography of monosaccharides from the lipid-free walls demonstrated only arabinose and galactose, in an estimated ratio of 4:1. Mannose and glucose, which could have been separated in the systems used, were not observed.

The bound lipids isolated after treatment with KOH in ethanol were not homogeneous (Fig. 13). One component, having similar properties to an authentic sample of mycolic acid from Mycobacterium tuberculosis (kindly supplied by Dr N. Polgar), was isolated and methylated with diazomethane. After methylation at least two new components were observed on chromatograms. A mass-spectrum of the methylated component confirmed that it was a mixture, but that the maximum molecular weight was less than 600.

Phenol extraction. Two samples of walls, freed of 'extractable' lipids, were treated with 90% phenol. Of the original material 96% and 98% respectively was insoluble. The phenol-soluble, water-insoluble fraction contained about 4% of neutral sugar (corresponding to 0.1% of the walls) and the phenol- and water-insoluble material about 11% (0.4% of the walls). It was clear that no major component was separable from the walls using phenol.

Direct extraction with KOH in ethanol. Some batches of walls were extracted directly with ethanolic KOH without the previous removal of lipid or membranes. Subsequent treatment with chloroform–methanol (2:1 by vol.) removed between 40% and 70% of material, leaving residues of rather variable composition, approaching that of the lipid-free walls prepared by the more elaborate method. An amino acid analysis of one sample showed

Table 2. Composition of walls before and after removal of lipids

<table>
<thead>
<tr>
<th>Composition expressed as percentage of the dry weight of each type of material, with standard deviations. Figures in brackets are numbers of batches used, where more than one.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative weights obtained</td>
</tr>
<tr>
<td>100</td>
</tr>
<tr>
<td>78</td>
</tr>
<tr>
<td>46</td>
</tr>
<tr>
<td>SDS-treated walls</td>
</tr>
<tr>
<td>DOC-treated walls</td>
</tr>
<tr>
<td>Walls freed from extractable lipids</td>
</tr>
<tr>
<td>Lipid-free walls</td>
</tr>
<tr>
<td>Extractable lipids</td>
</tr>
<tr>
<td>22 ± 5 (8)</td>
</tr>
<tr>
<td>0.21</td>
</tr>
<tr>
<td>0.21 ± 0.06 (4)</td>
</tr>
<tr>
<td>0.14</td>
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<tr>
<td>Bound lipids</td>
</tr>
<tr>
<td>41 ± 5 (5)</td>
</tr>
<tr>
<td>8.9</td>
</tr>
<tr>
<td>14.6 (2)</td>
</tr>
<tr>
<td>Neutral sugarb</td>
</tr>
<tr>
<td>25 (2)</td>
</tr>
<tr>
<td>25</td>
</tr>
<tr>
<td>28:5</td>
</tr>
<tr>
<td>54 (2)</td>
</tr>
<tr>
<td>Galactose</td>
</tr>
<tr>
<td>7:3 (2)</td>
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<tr>
<td>7:8</td>
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<tr>
<td>8:9</td>
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<tr>
<td>14:6 (2)</td>
</tr>
<tr>
<td>Glucosamine</td>
</tr>
<tr>
<td>3:5</td>
</tr>
<tr>
<td>7:6</td>
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<tr>
<td>3:9</td>
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<tr>
<td>Muramic acid</td>
</tr>
<tr>
<td>3:5</td>
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<tr>
<td>7:6</td>
</tr>
<tr>
<td>3:6</td>
</tr>
<tr>
<td>Galactosamine</td>
</tr>
<tr>
<td>Methyl pentosef</td>
</tr>
<tr>
<td>3:0</td>
</tr>
<tr>
<td>1:4</td>
</tr>
</tbody>
</table>

* Not measured;  \( ^b \) as galactose;  \( ^c \) Blix method after 24 h.;  \( ^d \) Blix method;  \( ^e \) amino-acid analyser, after 16 h. hydrolysis. Probably low;  \( ^f \) as rhamnose.
Fig. 13. Tracing of thin-layer chromatogram of bound lipids from walls of *Mycobacterium leprae-murium*. Samples were: 1, methylated mycolic acid from *M. tuberculosis*; 2, bound lipids; 3, methylated sample of shaded component; 4, partially saponified methylated mycolic acid.

substantial traces of 'nonmucopeptide' amino acids. This method was used by Cummins *et al.* (1967) to prepare one of their wall samples.

*Further treatment of lipid-free walls.* When lipid-free walls were treated with 0·1 N-\(\text{H}_2\text{SO}_4\) at 37°, progressive release of carbohydrate occurred. The material released contained arabinose and galactose, observed by thin-layer chromatography, and the ratio of galactose to total carbohydrate released was similar at 24, 48 and 72 h. (and similar to that in the lipid-free walls) suggesting that the sugars occurred in a single type of polysaccharide. In 3 days dilute acid made soluble 43% ± 4 (S.D.) of the original material from 4 batches, corresponding to about 70% of the nonmucopeptide carbohydrate. Most of the phosphorus remained in the insoluble portion. Sodium hydroxide (0·1 N) at 37° had little effect on the lipid-free walls (Table 3).

The released carbohydrate could be isolated after freeze-drying as a very hygroscopic white powder. One batch contained neutral sugar (80%), galactosamine (0·9%) and glucos-

<table>
<thead>
<tr>
<th>Composition of walls treated with 0·1 N-(\text{H}_2\text{SO}_4) or -(\text{NaOH}) at 37° for 3 days</th>
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</thead>
<tbody>
<tr>
<td>Lipid-free walls</td>
</tr>
<tr>
<td>Amount</td>
</tr>
<tr>
<td>Phosphorus</td>
</tr>
<tr>
<td>Neutral sugar*</td>
</tr>
<tr>
<td>Galactose</td>
</tr>
<tr>
<td>Total amino acids*</td>
</tr>
</tbody>
</table>

* As galactose; * Including hexosamines from amino acid analyses.
amino (0.5%), which corresponds to 37%, 58% and 7% of the amounts of these components in the original lipid-free walls.

Amino acids and hexosamines, consisting of alanine, glutamic acid, diaminopimelic acid, glucosamine and muramic acid in molar ratios 1:3:0:8:1:0:0:5:0:3 made up 42% of the residue of one batch after 3 days acid treatment. There were traces of a few other amino acids, mainly leucine, in a molar ratio of 0.07. The material contained almost all the glucosamine and about half the galactosamine of the original lipid-free walls.

The acid-treated residue was fairly resistant to lysozyme. The lysozyme-insoluble material was 80% of the original; about 6% and 3% of the total glucosamine appeared, respectively acetylated and free in the supernatant.

The lipid-free walls – after treatment with dilute acid – were dinitrophenylated. Mono-DNP-diaminopimelic acid was isolated as the main dinitrophenyl-amino acid.

No carbohydrate was released into the supernatant during 24 h. treatment with phosphodiesterase.

**Electron microscopy after extractions.** Removal of ‘unbound’ lipids had no effect in the appearance of sections of the walls. Treatment with 0.5% KOH in ethanol left the two-layer appearance unaffected, but seemed to destroy the stiffness of the walls, so that they collapsed (Fig. 9, 10). Subsequent extraction with chloroform–methanol left only the single electron-dense layer (Fig. 11). In two experiments using the silver methenamine stain, much more silver was deposited on the wall before than after removal of carbohydrate with dilute acid. Fig. 12 shows the appearance of the silver stain. The technique was rather capricious, both as to the size of silver grains produced and as to the reduction in silver staining after acid treatment, but the carbohydrate did not appear to be present as a separate layer in the walls. Ruthenium red also stained the lipid-free walls.

**DISCUSSION**

A fairly simple basic structure emerges from published data on walls of both pathogenic and other mycobacteria, namely a basal mucoprotein layer (Takeya & Hisatsune, 1963; Takeya, Hisatsune & Inoue, 1963), containing N-acetylglucosamine and N-glycolylmuramic acid (Adam et al. 1969; Azuma et al. 1970), alanine, glutamic acid and diaminopimelic acid, arranged as in the cell walls of other bacteria (Petit, Adam, Wietzerbin-Falszpan & Lederer, 1969; Wietzerbin-Falszpan et al. 1970) bearing a glycolipid. The polysaccharide is attached by phosphodiester bonds (and possibly also by other bonds) to the 6-position of the muramic acid (Kanetsuna, 1968; Kanetsuna & San Blas, 1970). The major polysaccharide is an arabinogalactan (Misaki & Yukawa, 1966), and is esterified with the characteristic long-chain mycolic acid on terminal arabinose units (Azuma, Yamamura & Fukushi, 1968; Azuma, Yamamura & Misaki, 1969; Kanetsuna, Imaeda & Cunto, 1969; Amar-Nacash & Vilkas, 1970).

Some species appear also to contain other polymers, particularly galactomannans and glucans, but it is not clear that these are covalently attached to the wall. Imaeda, Kanetsuna & Galindo (1968) have suggested for the walls of several mycobacterial species a structure containing multiple layers of such components, as well as the basic structure.

When first isolated the walls of *Mycobacterium lepraemurium* contain much membrane material. This is probably attributable to the method of preparation, which leaves the walls almost intact except for a single break. The membrane material is readily removed by detergents which lyse bacterial membranes, provided this is done before treatment of the walls with organic solvents. It is not removed by trypsin digestion.
When membrane and 'extractable' lipids are removed, so that the residue is free from substances that can be removed without breaking covalent bonds in the wall structure, the composition and properties of the walls resemble those of the 'basic' mycobacterial wall rather closely. Arabinose and galactose are the main neutral sugars present. Glucose found in the crude walls probably represents bacterial glycogen (Chargaff & Moore, 1944) and mannose occurs in mannophospholipids (Lee & Ballou, 1965). Methyl pentose is present in the crude walls, and possibly also in the lipid-free walls, though there is some uncertainty in the latter case because of the large correction that must be applied for other sugars present that interfere with the colour reaction. In the crude walls the methyl pentose probably occurs as traces of the fibrillar capsule material recently described (Draper & Rees, 1970), which contains 25% of methyl pentose as its sole carbohydrate (P. Draper, unpublished results).

Removal of bound lipid with ethanolic alkali, followed by chloroform-methanol, alters the ultrastructural appearance of sections of the walls, and their apparent flexibility, and allows the carbohydrate to be removed by gentle treatment. Takeya & Hisatsune (1963) described the removal of 'paired fibrous structures' from the surface of Mycobacterium phlei with ethanolic alkali. The bound lipid in M. lepraemurium was said by Kanetsuna, Imaeda & San Blas (1968) to be 90% mycolic acid, with an infrared spectrum identical to that of authentic mycolic acid. In our strain this seems not to be so; the bound lipid is a mixture of substances, none of which has the very high molecular weight of mycolic acid. It is, however, attached with an alkali-labile (presumably ester) bond. It forms the outer electron-transparent layer of the wall, which disappears when the lipid is removed. The lipid-free walls correspond to the basal layer from M. phlei of Takeya, Hisatsune & Inouye (1963).

Hughes & Tanner (1968) showed that carbohydrate attached to mucopeptide by a phosphodiester link is readily removed by dilute alkali and less readily by dilute acid. Although mycobacteria probably contain such a linkage, since muramic acid 6-phosphate can be isolated from them (Liu & Gotschlich, 1967; Kanetsuna, 1968), acid but not alkali removes the carbohydrate from Mycobacterium lepraemurium. The alkali-stability of the link implies that the sugar next to the phosphate does not have a free hydroxyl group in the 2-position. One may speculate that the small amount of galactosamine which persists in the walls, and of which half is found in the carbohydrate released by acid, may be the sugar involved; phosphorus and galactosamine occur in roughly equimolar amounts in the lipid-free walls.

No feature of the walls of Mycobacterium lepraemurium so far discovered explains their especial resistance to lysis by the enzymes of lysosomes to which they are exposed in the host cell (Brown, 1970; Brown & Draper, 1970), the only environment in which they will grow. The bound lipid may differ from that of other species, and they seem more resistant to lysozyme than has been reported of other species (cf. Kanetsuna, 1968; Amar-Nacash & Vilkas, 1969). In fact the organisms are apparently mainly protected by a loosely attached lipoglycopeptide, which is shed during isolation of the walls (Draper & Rees, 1970).

I am grateful to many colleagues for advice and discussion; in particular to Mr I. Burdett for photographing Fig. 6, 7 and 8, Dr D. Calam for the mass spectroscopy, Mr N. Gregory for gas chromatography of sugars, Dr S. Jacobs for amino acid analyses, Mrs S. Payne for chromatography of lipids, and Dr R. J. W. Rees and Mr C. Gibbs for providing material from infected animals.
Walls of Mycobacterium lepraemurium

REFERENCES


