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Chemical Composition of the Cell Wall of the H37Ra Strain of *Mycobacterium tuberculosis*

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The cell wall of the H37Ra strain of *Mycobacterium tuberculosis* was isolated and freed of extraneous noncovalently linked material by a series of extraction and enzymatic procedures. Chemical analysis of the cell wall has revealed the following composition: 22.8% amino acids, principally alanine, glutamate, and diaminopimelate in a molar ratio of 1:1.8:0.8; 24.7% reducing sugars, all in the form of arabinose and galactose in a molar ratio of 2.6:1; and 3.95% amino sugars, all in the form of glucosamine, muramic acid, and galactosamine in a molar ratio of 1:6.6:0.8. About 32.1% of the dry weight of the cell wall is lipid, of this about 55% is in the form of two series of mycolic acids. Each series of mycolic acids contains two homologues differing by 28 mass units. One pair of homologues contains in each a carbonyl function and an unsaturated double bond; the other pair contains two cyclopropane groups in each homologue. The remaining lipids are composed principally of normal saturated fatty acids, including tuberculostearic acid.

Cell walls have been prepared from several strains of mycobacteria and partial analyses of these cell wall preparations have been reported (8, 17, 18). Two of these reports include data on the H37Ra strain of *Mycobacterium tuberculosis*. Belknap, Camien, and Dunn (6) and Migliore, Acharya, and Jollès (18) reported on the amino acid content of the cell wall of H37Ra; their results differed considerably.

As part of our continuing investigations on structure and biosynthesis in the H37Ra strain, it was important to have available complete and accurate information on the composition of the cell wall of H37Ra. In this report we describe the large-scale preparation of cell wall from H37Ra and the chemical composition of these walls. We have accounted for some 83% of the dry weight of the cell wall which, in its isolated form, is more complex than a mucopeptide. Nonmucopeptide material is firmly bound to the basic structure of the cell wall and, operationally, must be considered to be a part of the cell wall.

METHODS AND MATERIALS

Preparation of crude cell walls of H37Ra. H37Ra cells were grown, harvested, and washed as previously described (14). The cells were disrupted (14) in an atmosphere of N_2 for 30 min at 2 to 9 C in a colloid mill with glass beads, by using a buffered medium containing 10 mM phosphate (*p*H 7.0) and 1.0 mM dithiothreitol. The glass beads and intact cells were allowed to settle, and the turbid suspension was centrifuged at 3,000 × *g* for 30 min; the residue was set aside. The supernatant solution was centrifuged at 10,000 × *g* for 30 min; the residue from this step was combined with the residue from the first centrifugation step and served as the source of crude cell walls and intact cells.

The cell wall-intact cell residue from the first grinding was reground with glass beads as before. The buffer was 10 mM potassium phosphate (*p*H 7.0) and 1.0 mM $MgCl_2$. The $MgCl_2$ was incorporated for other reasons, in this case for isolation of cytoplasmic membrane particles. It is not pertinent to the cell wall preparation. Grinding was for 60 min at 2 to 9 C. The beads and any residual intact cells were permitted to settle out and were discarded. Crude cell walls were recovered by differential centrifugation at 10,000 and 25,000 × *g*. The crude cell wall material was lyophilized and treated with trypsin. The dried cell wall material was suspended in 200 ml of tris(hydroxymethyl)aminomethane (Tris)-chloride buffer (0.05 M, *p*H 8.0) containing 400 mg of trypsin. The suspension was incubated at 37 C for 2 hr. The residue was recovered by centrifugation, washed five times with distilled water, and then lyophilized.

Purification of cell walls. The lyophilized product (10 g) was refluxed with 600 ml of ethanol-diethyl ether (1:1) for 1 hr; the residue was allowed to settle, and the colored supernatant solution was removed. This extraction was repeated (four or five times) until evaporation of the solvent showed that no further material was extracted. The residual material was repeatedly refluxed with 300 ml, each time of chloro-

form for 2 to 3 hr. The chloroform extraction was repeated until evaporation of the solvent showed no further soluble material being removed. The solvent-extracted residue was then treated for 1 hr with 90% (w/w) phenol-water (15). The suspension was centrifuged at 35,000 × *g*, the supernatant fraction was discarded, and the sediment was treated a second time with 90% phenol. The phenol-insoluble residue was recovered, dialyzed, washed, and lyophilized. The dried residue was treated with trypsin, chymotrypsin, and pepsin by the method of De Wijs and Jollès (8) as the final step in the preparation of pure cell walls.

In an alternative procedure, the freshly harvested cells were solvent-extracted before disintegration. One hundred grams of cells were refluxed three times, 3 hr each time, with 400 ml of ethanol-diethyl ether (1:1, v/v). The residue was then refluxed three times, for 4 hr each time, with 300 ml of chloroform. All solvent washes were discarded.

The extracted cells were disintegrated in a small colloid mill. Two grams of cells and 15 ml of glass beads were suspended in 30 ml of 0.4% sodium dodecyl sulfate and broken. The crude cell wall fragments were isolated by differential centrifugation at 10,000 and 25,000 × *g*. The crude cell wall residue was washed twice with water and then processed through the phenol and proteolytic enzyme treatments as described above.

Analytical methods: amino acids. The molar ratios of the amino acids were determined with a Beckman model 120 amino acid analyzer. The cell walls were hydrolyzed in 6 N HCl for 24 hr at 110 C. HCl was removed by repeated evaporation-resolution in water. The dried hydrolysate was freed of noncovalently bound fatty acids and mycolic acids by extraction with diethyl ether; the extracted residue was dissolved in water for analysis.

Amino sugars. The cells were hydrolyzed in 2 N HCl for 4 hr at 110 C. The hydrolysate was freed of HCl and freed of lipid with diethyl ether. Muramic acid was separated from glucosamine and galactosamine by electrophoresis on Whatman 3MM paper in a buffer of water-pyridine-acetic acid (300:27:12, v/v) at *p*H 6.5 (2,500 v, 150 ma, 45 min).

Glucosamine and galactosamine were separated either by preparative paper chromatography on Whatman no. 1 paper in a solvent system of butanol-pyridine-water (6:4:2, v/v) or by ion exchange chromatography on an Amberlite GC 120 X 8 [200 to 400 mesh (8)] with 0.33 N HCl as eluting agent (7).

The three amino sugars were separately estimated by the Rimington (19) modification of the Elson and Morgan (10) procedure by using glucosamine-HCl as an internal standard.

Reducing sugars. The cell walls were hydrolyzed in 1 N HCl for 2 hr at 110 C. The sugars were separated and identified by paper chromatography in a solvent system of butanol-pyridine-water (5:3:2, v/v).

Arabinose was determined by the orcinol procedure of Dische (9); galactose does not interfere. Galactose was determined by the Galactostat system of Worthington; arabinose does not interfere.

Lipids. Cell wall (1.0 g) was saponified in ethanolic-KOH for about 4 hr. The suspension was cooled, acidified, and extracted three times, each time with 150 ml of diethyl ether. The ether extracts were combined, washed with water, and then dried over anhydrous sodium sulfate. The diethyl ether was removed by evaporation. The residue was refluxed twice, each time for 30 min, with 200 ml of methanol. The hot methanol-soluble portion (normal fatty acids) was decanted and recovered by evaporation. The hot methanol-insoluble portion (mycolic acids) was dried. The lipids were esterified with freshly prepared diazomethane.

Mycolic acids. The methyl esters of the mycolic acids were purified by chromatography on a column (0.8 by 39 cm) of silica gel, by using as eluent a mixture of ligroin, benzene, and diethyl ether in the order of increasing polarity. The fractions containing mycolic acids were identified by silica gel thin-layer chromatography by using a solvent system of 95% petroleum ether and 5% diethyl ether. Infrared and nuclear magnetic resonance (NMR) spectra of the methyl esters of mycolic acids were taken on a Beckman model IR-5 spectrophotometer and a Varian Associates model T-60 NMR spectrometer, respectively. Mass spectra of the methyl mycolates were taken on an MS-9, double-focus, high-resolution mass spectrometer at temperatures of 260 to 300 C.

Normal fatty acids. The methyl esters of normal fatty acids were identified on a gas-liquid chromatographic apparatus (Barber-Colman, series 5000), by using 6% silicone on a Chromosorb W column (48 by 0.19 inch). The column temperature was programmed from 100 to 270 C. Standard methyl esters of normal fatty acids were obtained from Applied Science Laboratories.

Electron microscopy. Cell wall preparations were examined in the electron microscope by James Perdue of the Institute for Enzyme Research and Irving Sachs of the Forest Products Laboratory, Madison, Wis. Specimens for observation with the transmitting electron microscope were carbon-shadowed at a low angle (about 5°). The specimens were then observed with an RCA Universal 3D electron microscope. Specimens for observation with the scanning microscope were coated with a 10 to 20 nm layer of gold and viewed in a Cambridge Stereoscan scanning microscope.

RESULTS

One kilogram of wet cells yielded, after double breaking, differential centrifugation, and initial trypsin treatment, about 10 g of crude cell wall material which resembled the appearance of a cell wall when viewed in the electron microscope (Fig. 1).

After this material was subjected to a series of solvent extractions with alcohol-diethyl ether, chloroform, and phenol, and to a second enzymatic treatment with trypsin, chymotrypsin, and pepsin, about two-thirds of its contents were released into solution, leaving about 3.5 g of pure

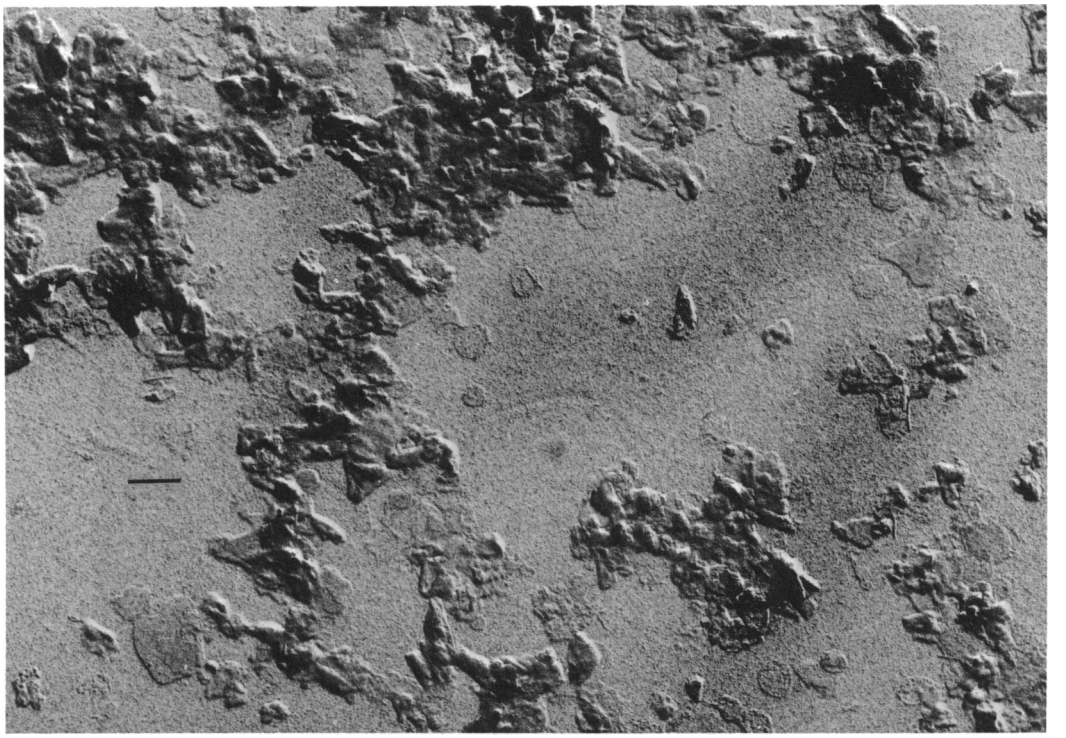


FIG. 1. *Electron micrograph of carbon-shadowed crude cell wall material before solvent extraction. Marker indicates 500 nm.*

cell wall (Fig. 2). Some of the solubilized constituents which have been identified include about 360 mg of phospholipids, 50 mg of Wax A, 5 mg of Wax C, 39 mg of Wax D, and 650 mg of phenol-soluble lipoproteins. Approximately 2.3 g of unidentified substances went into solution on final treatment with trypsin, chymotrypsin, and pepsin. In the second method of cell wall preparation, 100 g of freshly harvested wet cells yielded about 70 mg of cell walls.

Amino acids. The three major amino acids of the cell wall of H37Ra, as determined by the amino acid analyzer, are alanine, glutamic acid, and diaminopimelic acid, which are present in a molar ratio of 1.0:1.83:0.83, respectively. Glycine and aspartic acid are also present but in considerably smaller amounts. Small quantities of threonine, serine, valine, and phenylalanine are present; their presence has been reported earlier and their role is not clear. Amino acids account for 22.8% of the dry weight of the cell wall.

Optical form of the amino acids. All the diaminopimelic acid is present in the meso form. L-Glutamic acid was determined with the nicotinamide adenine dinucleotide-specific L-glutamic acid dehydrogenase; the ratio of D-glutamic to L-glutamic was 1:1. L-Alanine and D-alanine were determined specifically with the L-alanine-α-ketoglutaric transaminase and the D-amino acid oxidase systems (12). When the alanine fraction of the total hydrolysate was isolated by either ion exchange or preparative paper chromatography, only 32% of the material assayed for L- plus D-alanine. Enzymatic analysis yielded a ratio of L-alanine to D-alanine of 1.94. Identical results were obtained by gas-liquid chromatography (GLC) of the *N*-trifluoroacetyl-*O*-isopropyl derivatives of amino acids on a *N*-trifluoroacetyl-L-valyl-L-valine-*O*-cyclohexyl ester-coated capillary column (13). Both the true alanine percentage and the L to D ratio were confirmed in this method. The identity of the amino acid which is recognized as alanine by the ion-exchange-ninhydrin procedure of the amino acid analyzer, as alanine by paper and thin-layer chromatography but not by the specific enzymes and the gas-liquid chromatography technique is under investigation jointly here and in the laboratory of John Oró.

Reducing sugars. Only two sugars were found after hydrolysis of the cell wall; these were arabinose and galactose. The molar ratio of arab-

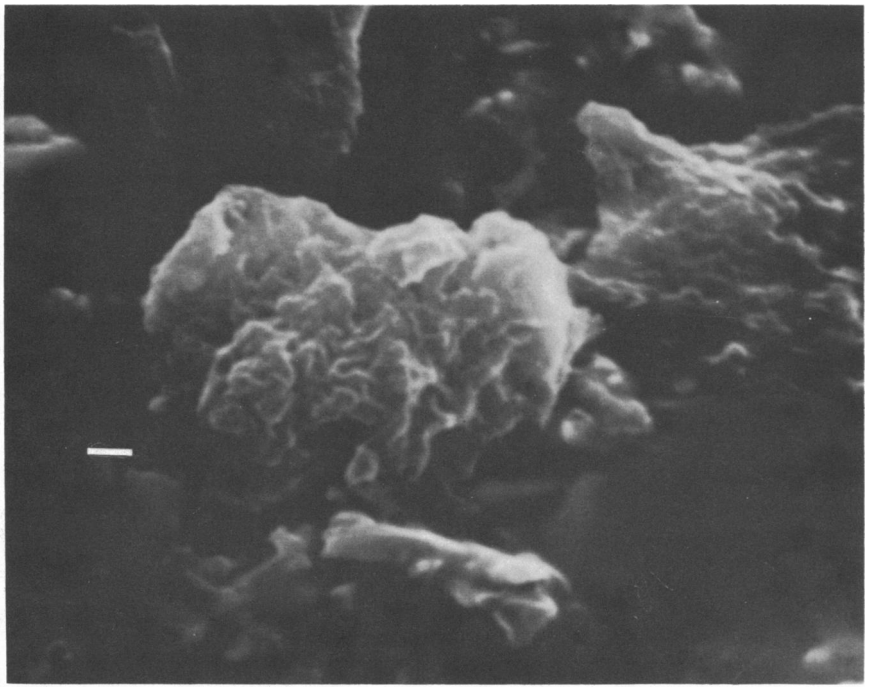


FIG. 2. *Scanning electron micrograph of gold-coated cell wall of M. tuberculosis after solvent extraction, phenol treatment, and treatment with proteolytic enzymes. The marker indicates 1,000 nm.*

inose-galactose is 2.6:1. Reducing sugars make up 24.7% of the dry weight of the cell wall.

Amino sugars. Glucosamine, muramic acid, and galactosamine are released by hydrolysis of the cell wall. These amino sugars account for 3.95% of the dry weight of the cell wall. The muramic acid showed a ratio of absorbance (505 to 530 nm after 18 hr) of 4.2 by the procedure of Elson and Morgan (10), confirming its identity and purity. Under the same conditions, the absorbance ratios for glucosamine and galactosamine were 0.89 and 0.74, respectively. No corrections were made for possible loss during hydrolysis. The molar ratio of glucosamine-muramic acid-galactosamine is 1:6.6:0.8. The same results were obtained by both methods of cell wall preparation.

Lipids. One gram of cell walls yielded, on total alkaline hydrolysis, 321 mg (32.1%) of lipid, of which 227 mg were insoluble in hot methanol and 94 mg were soluble. The insoluble fraction contained mycolic acids and the soluble fraction contained the normal fatty acids.

Mycolic acids. The hot methanol-insoluble fraction was esterified with diazomethane, and the resulting methyl mycolates were chromatographed on a silica gel column. The methyl mycolates were eluted with 40% ether plus 60% benzene and accounted for about 178 mg (78%). The product had a melting point of 58 to 60 C and moved as a large single spot on a silica gel plate in two different solvent systems: ligroin-diethyl ether, 95:5 and 92:8. Elemental analysis, in duplicate, of the product determined the carbon composition to be 82.39 and 82.19%, and that of hydrogen to be 13.26 and 13.29%. The infrared spectrum of the product shows a band at 1,025 cm^{-1} which can be attributed to the presence of cyclopropane rings, a band at 3,600 cm^{-1} owing to the chelated OH-group, and a strong ester band at 1,749 cm^{-1}.

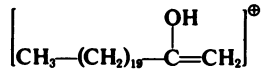
The NMR spectra of the mycolic acid were taken in two solvent systems: $CDCl_3$ and $CDCl_3$ plus one drop of D_2O. The NMR spectra revealed the presence of vinyl protons, a hydroxyl proton, methylene protons adjacent to a carbony-group, and the protons of cyclopropane rings.

The mass spectrum (Fig. 3) of the product shows that the product contains two series (A and B) of methyl esters of mycolic acids (Table 1). Each series contains two homologues. Each homologue in the same series differs from the other by 28 mass units.

As is generally the case with the mass spectra of methyl esters of mycolic acids, the molecular peaks of the four homologues do not show up in

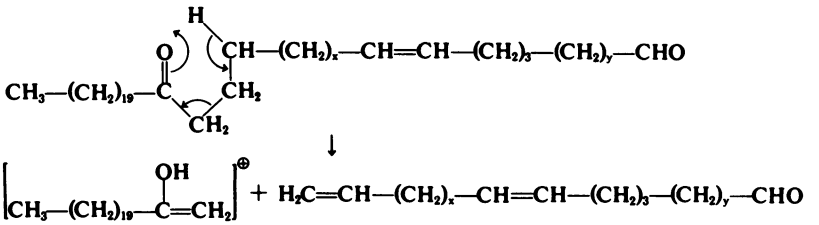
appreciable intensity. However, their methyl mycolate-50 (M-50) peaks are visible, more visible in series A than in series B. The prominent peak at m/e 410 shows that all four homologues yield methyl hexacosanoate on pyrolysis; this is typical of the methyl mycolates of virulent and avirulent strains of tuberculosis.

In series A, the mero-aldehydes do not show their molecular peaks; however, the mero-mycolal-18 peaks, m/e 752 and 780, are intense. Ion A, with a peak at m/e 325, is the typical ion common to both homologues of series A and is probably constructed as follows



m/e = 324

Ion A is formed by a rearrangement-fragmentation at the carbonyl group of the mero-aldehydes



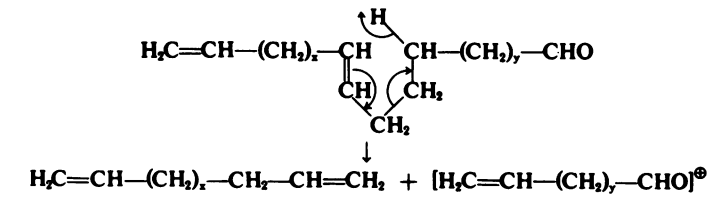
Ion A

The formation of the peak at m/e 325 rather than 324 is due to proton capture by ion A.

The remaining fragment of the mero-aldehyde

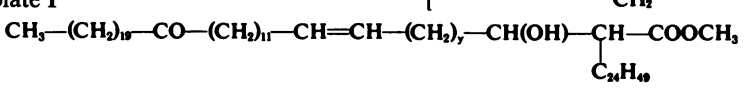
$H_2C{=}CH{-}(CH_2)_x{-}CH{=}CH{-}(CH_2)_3{-}(CH_2)_y{-}CHO$

undergoes further rearrangement and fragmentation:



Ions B

Fragments m/e 307 and 335 can be attributed to the ions B produced by the two homologues in the series A with values for y as 18 and 20, respectively. Each fragment has lost a proton to give the above peaks. The above considerations permit us to draw the structure of the methyl mycolates in series A as follows: (i) series A, methyl mycolate I



$y = 18$

$C_{80}H_{156}O_4$

and (ii) series A, methyl mycolate II, which is homologous with methyl mycolate I but two carbons longer

$y = 20$

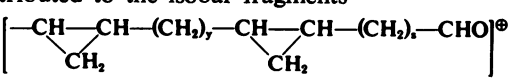
$C_{82}H_{160}O_4$

TABLE 1. *Characteristic m/e peaks of the mass spectrum of methyl esters of mycolic acids*

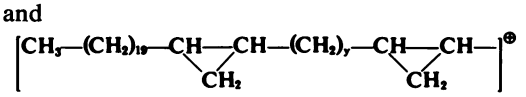
Ions	Series A		Series B	
	Homologue 1	Homologue 2	Homologue 1	Homologue 2
Methyl mycolates-50	1,130	1,158	1,128	1,156
Mero-mycolal			768	796
Mero-aldehyde-18	752	780		
Methyl hexacosanate	410	410	410	410
Isobar fragments of mero-mycolal			487	515
Ion A	325	325		
Ions B	307	335		

In series B, the mero-aldehyde peaks are at m/e 768 and 796. The mero-aldehyde-18 peak at m/e 750 peak is of appreciable intensity and that at m/e 778 is of very low intensity.

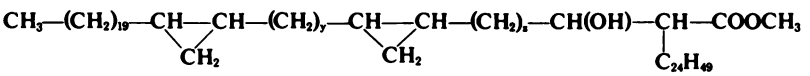
The two peaks m/e 487 and 515 can be attributed to the isobar fragments



and



The structure of the methyl mycolates in series B can, accordingly, be written: (i) series B, methyl mycolate I



$y + z = 27$

$C_{81}H_{158}O_3$

and (ii) the homologous series B, methyl mycolate II

$y + z = 29$

$C_{83}H_{162}O_3$

Normal fatty acids. The hot methanol-soluble fatty acids, mostly normal fatty acids, account for 22% of the total lipids of the cell wall. Gas-liquid chromatography of their methyl esters shows a range of chain length from C_{14} through C_{26} (Table 2). The major fatty acids, C_{16}, 10-methyl-C_{18}, and C_{26} are in the molar ratio of 2.7:1.1:0.67, respectively.

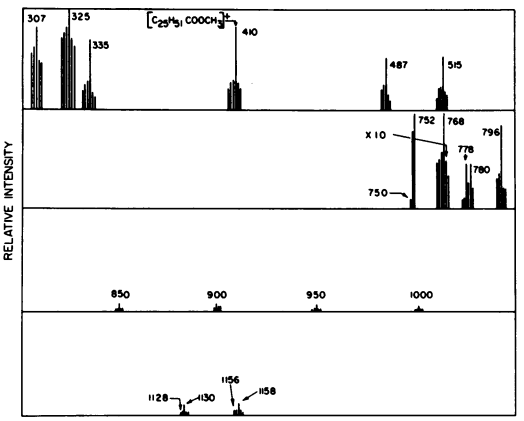


FIG. 3. *Mass spectrum of methyl β-mycolates. Only important peaks are represented in the figure. The spectrum was taken on MS-9 double-focus, high-resolution mass spectrometer at temperatures of 260 to 300 C.*

TABLE 2. *Molar ratios of mycobacterial cell wall fatty acids*

Carbon no.	Molar ratio
C_{14}	0.14
C_{16}	2.7
C_{18}	0.4
10-Methyl-C_{18}	1.1
C_{20}	0.18
C_{22}	0.11
C_{24}	0.10
C_{26}	0.67

DISCUSSION

The cell wall of H37Ra as finally isolated in this procedure is more complex than the usual bacterial peptidoglycan. This was expected from the reports of many investigators (1–5, 11, 16, 20), as far back as the work of Anderson and his associates thirty years ago, which showed that firmly bound lipids and glycolipids are characteristic of the cell wall of *M. tuberculosis*. Since each investigator used a specific procedural pattern in the isolation of mycobacterial cell wall and since most reports were relatively incomplete in terms of qualitative and quantitative data, it is most difficult and questionable to ascribe differences in composition of the cell wall to any specific characteristic, such as the strain, of the mycobacteria.

In this investigation, mycobacterial cell wall was prepared by two methods. In the first and routine method, the cells are ground and the cell hull is isolated and ground again to yield the crude cell wall preparation. Processing a large batch of material involved many operations over many days, and the possibility was considered that either intracellular enzymes or external contamination could introduce hydrolytic changes in the material, thereby giving us incorrect analyses on the final product. Although the latter source of contamination could be taken care of by strict control of the procedures, it was more difficult to rule out the possibility of release of destructive enzymes in the initial disruption of the harvested cells. This possibility was, however, ruled out by the preparative procedure which started with solvent extraction of the cells. The qualitative and quantitative results with cell walls prepared from the extracted cells agreed with the analytical results on the standard preparation.

The purpose of this investigation was to isolate and analyze a reproducible material which fulfilled the general definitions of a bacterial cell wall. The analysis will permit the continuation of work on the biosynthesis of both the cell wall and the macromolecules bound to it. The isolation of large amounts of mycobacterial cell wall will permit investigations on the location and type of linkage between the various macromolecules bound to the peptidoglycan.

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