MYCOBACTERIAL CELL WALLS

II. Chemical Composition of the "Basal Layer"

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Abstract

Takeya, Kenji (Kyushu University, Fukuoka, Japan), Kazuhito Hisatsune, and Yasuko Inoue. Mycobacterial cell walls. II. Chemical composition of the "basal layer." J. Bacteriol. **85**:24–30. 1963.—Chemical composition of the "basal layer" of the mycobacterial cell wall was determined. The layer contained 35% amino acids, 41.5% reducing sugars (mainly composed of arabinose and galactose), 13.8% amino sugars (glucosamine and muramic acid, 2:1), and 7.7% lipid. The main amino acids were alanine, glutamic acid, and diaminopimelic acid. Their molar ratio was approximately 2:2:1. The main difference in chemical composition between the cell wall and the basal layer was found in lipid content. According to the chemical composition, the basal layer resembles the walls of gram-positive bacteria, while the mycobacterial cell wall resembles the walls of gram-negative bacteria. The basal layer was thoroughly disintegrated by lysozyme digestion, and was considered to be an inner layer of the wall, conferring shape and rigidity on the mycobacterial cell wall.

In previous publications (Takeya et al., 1961*c*; Takeya and Hisatsune, 1963), it was shown that the thin "basal layer" of the mycobacterial cell wall, which appeared to be responsible for the shape and rigidity of the cell wall, was obtained by alcoholic KOH treatment of purified cell-wall preparations to remove the outer fibrous structure.

The present paper reports the chemical composition of the "basal layer."

Materials and Methods

Preparation of basal layer of the cell wall. The basal layer of the cell wall of *Mycobacterium phlei* was prepared by the method described in the accompanying paper (Takeya and Hisatsune, 1963). The layer was dried by lyophilization and used for further chemical analysis.

Estimation of amino acids. Samples were hydrolyzed by heating at 107 C in sealed tubes with 6 N HCl for 16 hr. Each hydrolysate was evaporated to dryness after filtering through a glass filter. The residue was dissolved in distilled water and again evaporated to dryness. This procedure was repeated four times. The final residue was redissolved in 1.0 ml of distilled water. Two-dimensional chromatograms were prepared by using Toyo-Roshi no. 51A (40 by 40 cm). *n*-Butanol–acetic acid–water (4:1:5, v/v/v) was used as the first solvent, and *m*-cresol–phenol–borate buffer (pH 9.3; 25:25:8, w/w/v) as the second solvent (Levy and Chung, 1953). After development, the chromatogram was washed twice with ether, dried, and then sprayed with about 3 ml of a mixture of acetic acid-ethanol (15:50, v/v). The amino acids were detected by spraying 0.02 to 0.2% ninhydrin solution in alcohol, followed by heating at 90 C for 5 to 10 min.

In addition to this, the hydrolysates were treated with dinitrofluorobenzene by the method described by Schroeder and LeGette (1953); resultant DNP-amino acids were also detected by two-dimensional paper chromatography.

For quantitative determination, the chromatogram was first sprayed with 0.02% ninhydrin in alcohol solution and heated at 75 C for several minutes. Each faintly colored spot was cut into small pieces, added to 0.2 ml of 0.1 N NaOH solution, and kept in vacuo on H_2SO_4 overnight. After extraction with 1 ml of distilled water, the amount of each amino acid was determined by colorimetry according to the method of Tsukamoto, Komori, and Inoue (1961). Nitrogen was determined by the micro-Kjeldahl method.

Estimation of amino sugars. Samples were hydrolyzed by heating at 100 C in sealed tubes with 4 N HCl for 10 hr. One-dimensional chromatograms were prepared, using *n*-butanol–

acetic acid–water (4:1:5, v/v/v), phenol saturated with water, or *m*-cresol–phenol–borate buffer (pH 9.3; 25:25:8, w/w/v) as solvents. The amino sugars were detected by spraying with Elson-Morgan reagent, aniline hydrogen phthalate, or periodic acid-benzidine reagents.

For quantitative determination of total content of amino sugars, hydrolysates were neutralized by NaOH solution after filtering through a glass filter. The content of amino sugars in the neutralized solution was determined by the Belcher, Nutten, and Sambrook (1954) modification of the Elson-Morgan reaction. Since some destruction of amino sugars would occur during hydrolysis of samples, corrections were made, using glucosamine as control.

For determination of the molar ratio of each amino sugar, the regions corresponding to spots on chromatograms were cut into small pieces and extracted with 2 to 3 ml of distilled water for 30 min at 100 C in an oil bath. After cooling, the amount of amino sugar in each extract was measured by the method of Belcher et al. (1954), as mentioned above.

Estimation of reducing sugars. Samples were hydrolyzed by heating at 100 C in sealed tubes with 2 N H_2SO_4 for 2 hr. After filtering through a glass filter, hydrolysates were neutralized with saturated barium hydroxide solution. The barium sulfate was centrifuged off, and the supernatant was evaporated to dryness in vacuo. From the resultant residues, reducing sugars were extracted with pyridine by the method of Malpress and Morrison (1949). Pyridine was distilled off in vacuo from the extract, and the residue was redissolved in a small amount of 10% isopropanol solution. The procedure for detecting sugars by chromatography was approximately the same as described for the estimation of amino sugars.

For quantitative determination of reducing sugars, the hydrolysates were neutralized with NaOH solution, after filtering through a glass filter. The content of total reducing sugars was determined by the method of Momose, Mukai, and Watanabe (1960), using 3,6-dinitrophthalic acid as the reagent. The molar ratio of each reducing sugar was determined by the method described above in the section on amino sugars.

Estimation of lipid content. Approximately 100 mg of samples were hydrolyzed by heating with 6 N HCl in sealed tubes at 105 C for 20 hr. The hydrolysates were extracted thoroughly with ether. Ether was evaporated, and the material dried in vacuo over P_2O_5 and NaOH; then the total ether-extractable lipid was determined by weighing.

Treatment of basal layer with enzymes. The basal layer was digested by lysozyme or nagarse, by the method described in the accompanying report in this series (Takeya and Hisatsune, 1963).

Results

An electron micrograph of the basal layer preparation used in this experiment is shown in Fig. 1.

Amino acids. The results obtained from quantitative estimation are shown in Table 1. The main components are glutamic acid, alanine, and diaminopimelic acid. Other amino acids were observed only in an amount less than 20% of the amount of diaminopimelic acid. They are aspartic acid, serine, glycine, threonine, valine, leucine or isoleucine, phenylalanine, histidine, and arginine.

The molar ratio of diaminopimelic acid, glutamic acid, and alanine was 1:1.7:1.9. The total amount of ninhydrin-positive substance was 33.0%, calculated as aspartic acid.

Amino sugars. Two spots corresponding, respectively, to glucosamine and muramic acid were detected. The absorption maximum of the extract obtained from each spot in the Belcher et al. (1954) modification of the Elson-Morgan reaction was 585 mμ and 510 to 520 mμ. These values are respectively consistent with those for muramic acid and glucosamine reported by Strominger, Park, and Thompson (1959). The molar ratio of glucosamine and muramic acid was 2.4:1.0, calculated as glucosamine. When corrected by the method of Strange and Dark (1956), the ratio became 1.8:1.0. The total amount of amino sugars was found to be 13.8% (Table 2).

Reducing substances. Two spots corresponding to arabinose and galactose were observed. The molar ratio of these sugars was tentatively determined to be 2:1. The total amount of reducing substances was 51.4%, calculated as glucose. Since the spot corresponding to galactose may also contain some unidentified sugars,

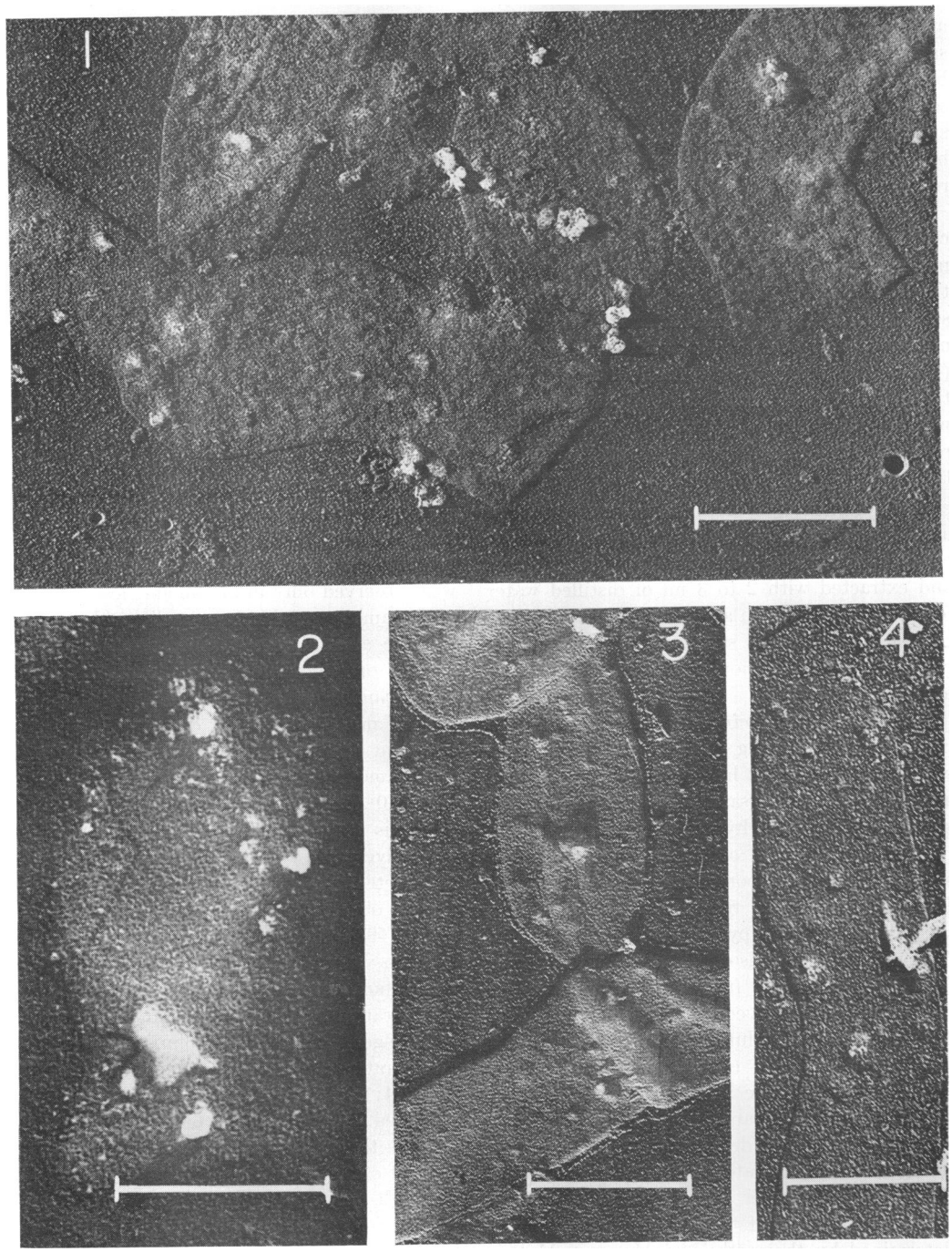

FIG. 1. *Basal layer preparation used in this experiment. The layers are rough-surfaced and appear to be covered with fine fibrils.* × *52,000. Magnification mark = 0.5 μ in each figure.*

FIG. 2. *Basal layer treated with lysozyme. Later stage of disintegration. Fine fibrils and tiny particles are left behind.* × *62,000.*

FIG. 3. *Basal layers treated with nagarse. The layers appear to become thinner and to be smooth-surfaced.* × *48,000.*

FIG. 4. *Smooth-surfaced basal layer.* × *47,000.*

TABLE 1. *Quantitative estimation of main amino acids of the "basal layer"*

Main components	Amount	Concn	Molar ratio
	mg/100 *mg*	μM	
Glutamic acid	10.7	72.7	1.7
Alanine	7.4	83.1	1.9
Diaminopimelic acid	8.1	42.6	1.0

TABLE 2. *Quantitative estimation of amino sugars of the "basal layer"*

Sugar	Calculated as glucosamine		Corrected by Strange and Dark (1956)
	Total	Molar ratio	Molar ratio
	mg/100 *mg*		
Glucosamine	13.8[a]	2.4	1.8
Muramic acid		1.0	1.0

[a] Glucosamine plus muramic acid.

TABLE 3. *Quantitative estimation of reducing sugars of the "basal layer"*

Total reducing sugars[a]	Reducing sugars except amino sugars[b]	Molar ratio of component reducing sugars	
		Arabinose	Galactose + unidentified sugars[c]
mg/100 *mg*	*mg*/100 *mg*		
51.4	41.5	2.0	1.0

[a] Calculated as glucose.
[b] Calculated as glucose.
[c] Calculated as galactose.

further examination will be necessary. The results are summarized in Table 3.

Lipids. The amount of lipids extracted by ether from a hydrolysate of the basal layer was estimated as 7.7%.

Treatment with lysozyme or nagarse. Treatment of the basal layer with lysozyme (1,000 μg/ml) at 37 C for 3 hr caused a thorough disintegration of the rigid structure. Thin original contours were gradually lost, leaving behind fine fibrils and tiny particles (Fig. 2). Reactions for amino sugars and reducing sugars were positive for the supernatant of the digest.

After digestion by nagarse (10,000 units/ml) at 37 C for 3 hr, the basal layer appeared to become thinner (Fig. 3), and reducing sugars were detected in the supernatant of the digest.

TABLE 4. *Comparison of the amino acid, ninhydrin-positive substance, nitrogen, reducing sugar, amino sugar, and lipid content of the cell wall and the "basal layer"*

Component no.	Component	"Basal layer"	Cell wall[a]
		%	%
1	Amino acid[b]	35.0	15.7
2	Ninhydrin-positive substance[c]	33.0	17.1
3	Nitrogen (N × 6.25)	5.8 (36.3)	2.6 (16.0)
4	Reducing sugar[d]	51.4	16.1
5	Reducing sugar[d] except amino sugar	41.5	14.3
6	Amino sugar[e]	13.8	2.3
7	Lipid	7.7	60.4
	Recovery (1 + 5 + 6 + 7)	98	93

[a] Hisatsune, Inoue, and Takeya (*unpublished data*).
[b] Sum of the each amino acid content estimated by quantitative method.
[c] Calculated as aspartic acid.
[d] Calculated as glucose.
[e] Calculated as glucosamine.

DISCUSSION

The results obtained in this experiment are summarized in Table 4, and compared with those on the cell wall obtained in a preliminary experiment (Hisatsune et al., *unpublished data*). The quantitative chemical compositions of cell wall of *M. phlei* coincide well with those of cell wall of the BCG strain of *M. tuberculosis*, reported by Kotani et al. (1959). The main difference in the chemical composition between the basal layer and the cell wall is found in lipid content.

A comparison of the chemical composition of the cell walls of gram-positive and gram-negative bacteria is presented in Table 5. In general, the walls of gram-negative bacteria are poorer in amino sugars and also often in reducing sugars, and richer in lipids. This difference is explained by the fact that the former contain a lipoprotein

TABLE 5. *Comparison of the reducing sugar, amino sugar, nitrogen, and lipid content of the various bacterial cell walls and "basal layer"*[a]

Cell wall	Reducing sugar	Amino sugar	N	Lipid
Gram-negative				
	%	%	%	%
Escherichia coli	16	3		22
Rhodospirillum rubrum	23	2		22
Pseudomonas aeruginosa	16–17	2.1–2.7		11
Salmonella pullorum	46	4.8		19
Vibrio metchnikovi	12	1.9		11
Mycobacteria				
BCG[b]	21	3.7	3	51.6
Mycobacterium phlei cell wall	16	2.3	2.6	60.4
M. phlei "basal layer"	51	13.8	5.8	7.7
Gram-positive				
Bacillus subtilis	34	8.5	5.1	2.6
Micrococcus lysodeikticus	45	16–18	8.7	2
Streptococcus faecalis	61	22	5.6	<2
S. pyogenes	55–62	18–22		0

[a] Data for gram-negative and gram-positive bacteria are collected from the reviews written by Salton (1960*a*, *b*).

[b] Kotani et al. (1959).

layer. The chemical composition of the basal layer, as a whole, resembles that of the walls of the gram-positive bacteria, whereas the mycobacterial cell wall resembles the walls of gram-negative bacteria in its content of amino sugars and reducing sugars. Enormous richness in lipid content is a characteristic feature of the mycobacterial cell wall, as previously reported by Kotani et al. (1959). From the electron microscope observations made by the present authors (Takeya et al., 1959; Toda et al., 1960; Koike and Takeya, 1961; Takeya et al., 1961*b*) of the ultrathin sections of mycobacteria, it is evident that the mycobacterial cell wall is multilayered, as seen in the case of the cell wall of *Escherichia coli* (Kellenberger and Ryter, 1958). The basal layer appears to correspond to the inner layer of the cell wall. The basal layer resembles the rigid layer of *E. coli* (Weidel, Frank, and Martin, 1960), since both of them are disintegrated by digestion with lysozyme, and the substances positive for the Elson-Morgan reaction are split off in the supernatant. Although only qualitative chemical analysis of the rigid layer was reported, its amino acid and amino sugar constituents are identical with those of the basal layer. Arabinose and galactose, the presence of which was considered to have taxonomic significance (Cummins and Harris, 1958), were found in the basal layer, whereas only glucose was present in the rigid layer.

The quantitative amino acid composition of cell walls from four strains of mycobacteria was reported by Belknap, Camien, and Dunn (1961). According to these workers, the approximate molar ratios of the main amino acids (i.e., glutamic acid, alanine, and diaminopimelic acid) were 2:2:1, 2:3:1, and 5:3:2 in *M. ranae*, the unclassified strain F811, and *M. tuberculosis*. The ratio was found to be 1.7:1.9:1.0 in the basal layer of the present experiment. The molar ratio of glucosamine and muramic acid was found to be approximately 2:1 for both mycobacterial cell wall and basal layer in this experiment, though the ratio had been reported to be 1:1 for the mucopolymer fragments, which represents an essential component of the rigid layer, obtained by lysozyme digestion of the cell wall of *E. coli* (Primosigh et al., 1961). The high content of reducing sugars in the basal layer may suggest the presence of macromolecular substance composed of arabinose, galactose, and unidentified sugars, which is comparable to the teichoic acid or teichuronic acid found in *Lactobacillus arabinosus* (Baddiley, Buchanan, and Cars, 1958), *Bacillus subtilis* (Armstrong et al., 1959; Janczura, Perkins, and Rogers, 1961), *Staphylococcus aureus* (Armstrong et al., 1959), and *B. megaterium* (Ghuysen, 1961). Teichoic acid from *S. aureus* was recently reported, by Haukenes et al. (1961) and Sanderson, Juergens, and Strominger (1961), to be serologically active. On the other hand, a glycopeptide fraction, containing arabinose and galactose, obtained from the culture filtrate of tubercle bacilli was identified as a split product from the cell wall, and was found to be highly potent in several immunological reactions (Takeya, Hisatsune, and Nakashima, 1961*a*). This may support the above

speculation. The lipid content of the basal layer obtained in the present experiment was comparatively high. However, further studies should be made to determine whether the lipid is an essential component of the basal layer, or a component removable by further treatment. Careful examination of the layer with an electron microscope revealed that some of the layers appeared smooth-surfaced (Fig. 4), while the surface of most layers appeared slightly rough and was supposed to be covered with fine fibrils (Fig. 1). On the other hand, extremely thin smooth-surfaced layers still having the original cell contour were obtained by the mechanical grinding of the basal layer with glass powders. From these facts, it may be plausible that the fine fibrils removable by mechanical means are mainly composed of lipid, and the high lipid content of the basal layer is not essential for preserving the rigidity of the layer.

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