CHEMICAL STRUCTURE OF THE CELL WALL OF MYCOBACTERIUM SMEGMATIS. I - ISOLATION AND PARTIAL CHARACTERIZATION OF THE PEPTIDOGLYCAN.

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SUMMARY. Cell walls of M. smegmatis were prepared by French press disruption and treatment with trypsin and chymotrypsin. Free lipids were extracted by organic solvents. Part of the bound lipids were solubilized by an alkaline treatment and most of the remaining lipids and neutral sugars by mild acid hydrolysis, leaving an insoluble residue which has the composition of a peptidoglycan. This residue was solubilized by Myxobacter AL_1 enzyme. It was possible to isolate from the lysate a tripeptide Ala-Glu-DAP, three tetrapeptides Ala-Glu-DAP-Ala and also a tetrasaccharide and a disaccharide which give equimolar amounts of glucosamine and muramic acid after acid hydrolysis. The disaccharide differs from the typical β-1,4-N-acetylglucosaminyl-N-acetylmuramic acid by its Rf values in various solvents.

Previous work on the chemical structure of the mycobacterial cell wall (1-10) showed that it contains a peptidoglycan and an arabinogalactan polymer esterified by mycolic acids. The link between the peptidoglycan and the arabinogalactan polymer might be through phosphodiester bonds, as suggested by the isolation of 6-phosphomuramic acid (9). Mycolic acids are, at least in part, attached to the 5-position of arabino-furanose residues of the arabinogalactan (11,14).

Our aim was to sequentially solubilize the components of the cell wall in order to study their structure and the linkage between them. In the experiments described in this paper we isolated the peptidoglycan which was then solubilized with Myxobacter AL_1 enzyme (12). Its major structural components : tetra- and tri-peptides, di- and tetra-saccharides were isolated.

Preparation of the cell walls. Cells were grown in Roux bottles on Sauton's medium for 12 days at 37°, harvested by filtration and washed with distilled water. Cells (300 g, wet weight) were suspended in distilled water (1.5 l) with a blendor and disrupted at 4° with a French press at 5.000 psi (two successive runs). DNase was added after the first and the second run to decrease the viscosity. The suspension was then diluted three times with water and the unbroken cells were removed by three successive centrifugations

at 800 g, for 15 min. at 4°. The resulting supernatant was centrifuged at 27.000 g for 45 min. and yielded a pellet of cell walls which was resuspended in three liters of 0.067 M phosphate buffer pH 7.8 ; trypsin and chymotrypsin (750 mg each) and a few drops of toluene were added. After incubation overnight at room temperature, the cell walls were recovered by centrifugation and washed, by resuspension and centrifugation, three times with phosphate buffer and three times with distilled water. The yield was 7.4 g of cell walls (dry weight) ; this is a minimum yield as the cell walls tend to aggregate and a part of them was eliminated with the unbroken cells.

These cell walls were delipidated at room temperature by repeated extraction with acetone, alcohol-ether (1:1), chloroform and chloroform-methanol (2:1) which removed respectively 0.665 g, 0.480 g, 0.725 g and 0.420 g of free lipids. The chemical composition of the delipidated cell walls in given in Table I.

TABLE I

Composition of the cell walls of M. smegmatis at various stages of the degradation.

	Lipids	Neutral sugars*	Amino sugars**	Amino acids***	Phosphorus
Delipidated cell walls	33 %	30 %	8.5 %	12.4 %	0.07 %
" after KOH treatment	21 %	38 %	9.7 %	18.8 %	0.09 %
" after H_2SO_4 treatment	8 %	4.9 %	22.4 %	49.5 %	0.16 %

* Arabinose and galactose (2:1).
** After hydrolysis, glucosamine and muramic acid (1:1).
*** Alanine, glutamic acid and DAP (1.6:1:1) + other amino-acids in minor amounts, mainly glycine and aspartic acid.

Isolation of the peptidoglycan. Delipidated cell walls were treated first with 0.5 % KOH in ethanol for 48 h at 37° according to Takeya and Hisatsune (6) and extracted with ether : this treatment resulted in the removal of part of the bound lipids (Table I). The residue was then heated in 0.1 N H_2SO_4 for 48 h at 60°, as proposed by Hall and Knox (13) and Acharya (14) : most of the remaining bound lipids and neutral sugars were removed by this treatment (Table I). To avoid a complete hydrolysis of the solubilized

polysaccharides, the sulfuric acid solution was removed by centrifugation and replaced by a fresh solution every hour for ten hours, then after 24 h. The acidic extracts were neutralized with baryum hydroxyde, filtered and lyophilized. Chromatography of the extracts on Whatman n° 1 paper in butanol-pyridine-water (5:3:2) showed the presence of polysaccharides of Rf = 0, of oligosaccharides and of arabinose and galactose. Some of the oligosaccharides could be isolated by gel filtration on Sephadex G 15 and purified by preparative paper chromatography : a di- and a tri-galactose and a substance moving faster than arabinose and giving only arabinose after acid hydrolysis were isolated.

The insoluble residue, obtained after alcoholic KOH and 0.1 N H_2SO_4 treatments, contains more than 90 % of the amino sugars, amino acids and phosphorus of the original cell walls. Its overall composition is that of a classical peptidoglycan (Table I).

Enzymatic solubilization of the peptidoglycan. The techniques used are those described in ref. 15. The peptidoglycan (300 mg) was incubated with 800,000 units of Myxobacter AL_1 enzyme in 0.01 M veronal buffer pH 9.0, the final volume being 70 ml (the activity of the Myxobacter AL_1 enzyme preparation was tested as described in ref. 16, using a suspension of Micrococcus lysodeikticus cells instead of Arthrobacter crystallopoietes). Lysis was complete in about 4 hours. Dissolution of the peptidoglycan was accompanied by the appearance (per mg of peptidoglycan) of 500 mμ equivalents of amino-groups of alanine, 50-80 mμ equivalents of mono-NH_2-DAP and of about 100 mμ equivalents of carboxyl groups of alanine. Thus, the main action of the enzyme seems to be the rupture of amide bonds at the junction between the glycan and the peptide parts of the peptidoglycan. Hydrolysis of DAP-Ala linkages seems also to occur.

Study of the peptidoglycan fragments. The lysate obtained by Myxobacter AL_1 enzyme action was filtered through two connected columns of Sephadex G 50 (h = 84 cm, ϕ = 3 cm) and G 25 (h = 86 cm, ϕ = 2.5 cm) (V_o = 390 ml ; $V_o + V_i$ = 880 ml) in 0.1 M LiCl. The elution pattern is given in Figure 1.

Peak M_1 contains most of the amino sugars of the cell wall. It was desalted by gel filtration, lyophilized and incubated with 1 mg of lysozyme in 30 ml of 0.03 M ammonium acetate, pH 6.2, for 24 h at 37°. The resulting products were filtered through a Sephadex G 15 column (h = 80 cm, ϕ = 1.5 cm) (V_o = 185 ml, $V_o + V_i$ = 300 ml) in 0.1 N acetic acid, giving the elution pattern showed in Fig. 2.

Fraction L_A contains the amino acids, the neutral sugars, most of the phosphorus and about one third of the total amino sugars present in peak

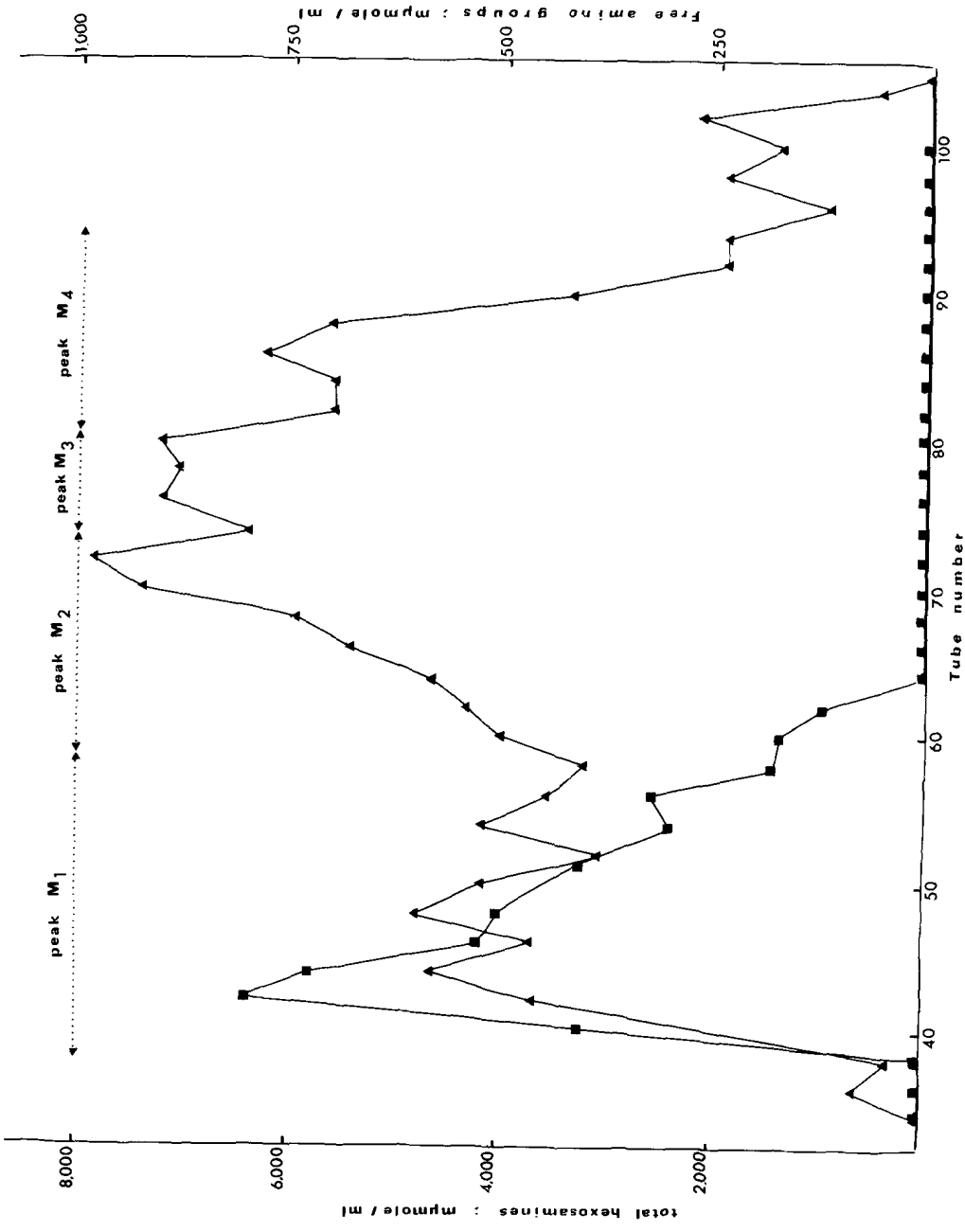

Figure 1 : Elution pattern of the Sephadex G 50 and G 25 columns.
-▲—▲- : free amino-groups ; --■—■-- : total hexosamines.
Volume of the fractions : 10 ml.

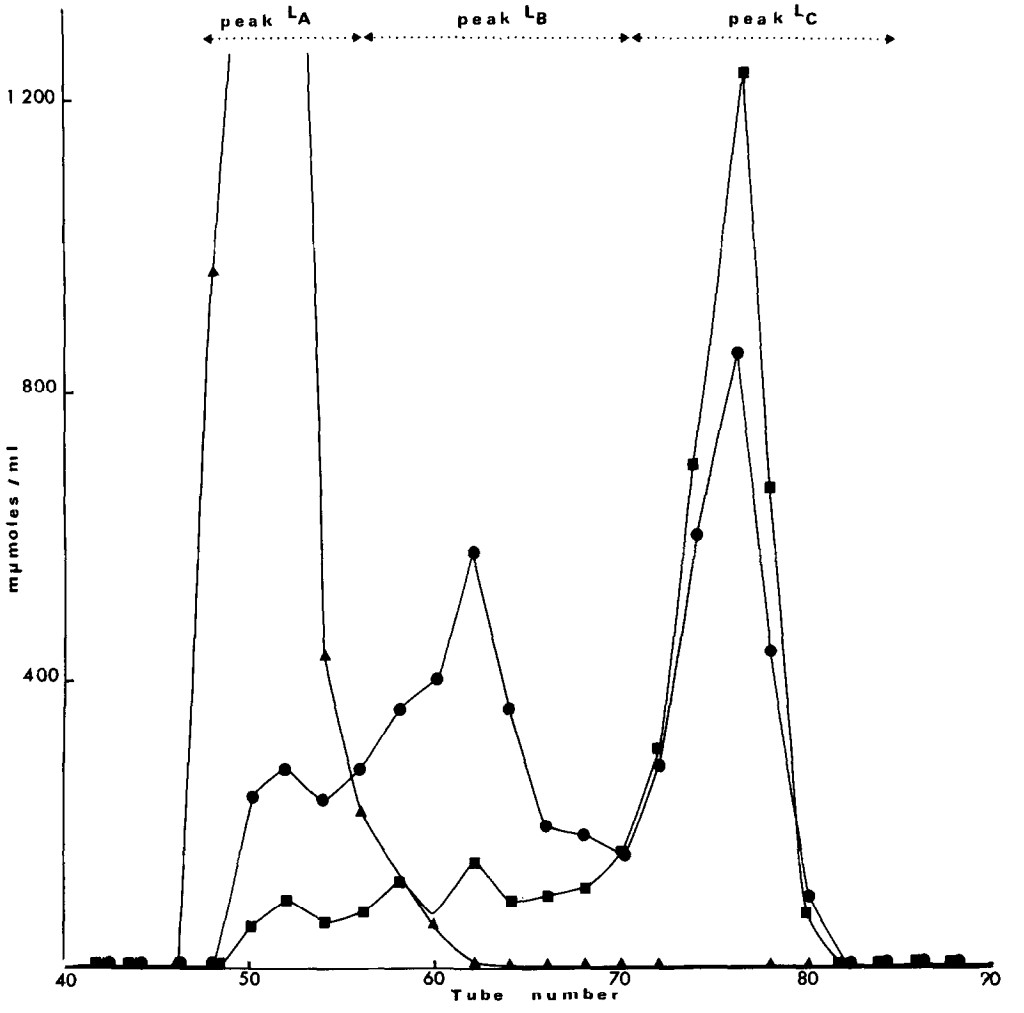

Figure 2 : Elution pattern of the G 15 column.

—▲— ▲— : free amino-groups ; —●— ●—: reducing groups ;
—■— ■— : Morgan-Elson reaction products after 30 min. of heating at 100° (15) expressed as N-acetylglucosamine.
Volume of the fractions : 3.7 ml.

M_1. Fraction L_B and L_C each contain about one third of the total amino sugars of peak M_1. The main components of fractions L_B and L_C (compounds B and C respectively) were isolated by preparative paper chromatography in n-butanol-acetic acid-water (5:1:2). After acid hydrolysis (HCl 4 N, 6 h, 100°) both gave rise to equimolar amounts of glucosamine and muramic acid. Reduction with $NaBH_4$ resulted in the disappearance of half of the muramic acid of B and all of the muramic acid of C. Moreover, by lysozyme treatment, B was partially hydrolyzed to C. Thus, B was identified as being a tetrasaccharide and C a

disaccharide. C was then compared with β-1,4-N-acetylglucosaminyl-N-acetylmuramic acid isolated from Micrococcus lysodeikticus (16-17-18). Both have the same spectrum in the Morgan-Elson reaction after 30 minutes of heating in alkalin buffer (15), but their chromatographic mobilities are different on Whatman n° 1 paper.

Solvent	$R_{N\text{-acetylglucosamine}}$	
	C	M. lysodeikticus disaccharide
n-butanol-acetic acid-water (5:1:2)	0.70	1.0
isobutyric acid-N-ammonia (10:6)	0.76	0.88

Enzymatic hydrolysis of C with Helix pomatia gut juice yielded N-acetylglucosamine (identified by paper chromatography in n-butanol-acetic acid-water and isobutyric acid-N-ammonia), and a second compound which gives muramic acid on acid hydrolysis but which is chromatographically different from N-acetylmuramic acid. Mass spectrometry suggests that the second compound is N-glycolylmuramic acid (19).

Acid hydrolysis of peak M_2 gave Ala, Glu and DAP in a molar ratio of 2:1:1.55. One third of the alanine and one fourth of the DAP have free amino groups. The material in peak M_2 seems rather complex and was not studied further.

Peak M_3 contained Ala, Glu and DAP in a molar ratio close to 2:1:1. Dinitrophenylation showed that half of the alanine was N-terminal and that about one fourth of the DAP had one free amino group. Peak M_3 seems to be a mixture of oligomers of the tetrapeptide : $(Ala\text{-}Glu\text{-}DAP\text{-}Ala)_n$, n having an average value of 4.

Peak M_4 was fractionated by filtration on Sephadex G 15 followed by high voltage preparative electrophoresis at 50 V/cm on Whatman n° 3 MM paper for 105 min. into fourmajor components. Three of them are tetrapeptides Ala-Glu-DAP-Ala and the last one is a tripeptide Ala-Glu-DAP as demonstrated by dinitrophenylation, hydrazinolysis and Edman degradation.

All of them give rise to ammonia after acid hydrolysis. Their electrophoretic mobilities were compared to that of a tetrapeptide Ala-Glu-DAP-Ala obtained by enzymatic degradation of Bacillus megaterium cell walls (20).

pH	Mobilities in cm						
	Ala	Glu	tetrapeptides				tripeptide
			B.megaterium	I	II	III	
1.9	-45	-29,3	-32.8	-49	-44.7	-36.4	-50.5
4.0	-5.4	+8.7	+ 6.4	-26.7	-12	-10	-27.7

After mild acid hydrolysis (HCl 4 N, 24 h, room temperature), electrophoresis of each of the tetrapeptide gave, among others, a spot having the same mobility as the Bacillus megaterium tetrapeptide. Thus the three tetrapeptides are probably amides of the tetrapeptide Ala-Glu-DAP-Ala. Tetrapeptide I seems to be diamidated, tetrapeptide II and III monoamidated, each on a different carboxyl group. The tripeptide has an electrophoretic mobility very close to that of tetrapeptide I. It should be diamidated.

Conclusion.

By combining mild chemical treatments and enzymatic hydrolysis all of the components of the cell wall of M. smegmatis were sequentially solubilized. By treating the cell walls with alcoholic KOH and diluted H_2SO_4, an insoluble residue was obtained which has the overall composition of a peptidoglycan. It was solubilized with Myxobacter AL_1 enzyme. Study of the action of the enzyme and of the structure of the fragments obtained gave a general view of the peptidoglycan structure. Its main components, tri- and tetrapeptides, di- and tetrasaccharide were isolated. The disaccharide differs from the classical disaccharide of M. lysodeikticus peptidoglycan in that the muramic acid residue is probably N-glycolylated instead of N-acetylated (19).

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References.

1 - C. Cummins and H. Harris, J. Gen. Microbiol., 18, 173, (1958).
2 - S. Kotani, T. Kitaura, T. Hirano and S. Tanaka, Biken's J., 2, 129, (1959).
3 - D.R. Belknap, M.N. Camien and M.S. Dunn, Proc. Soc. Exptl. Biol. Med., 107, 289, (1961).
4 - H. de Wijs and P. Jollès, Biochim. Biophys. Acta, 83, 326, (1964).
5 - K. Takeya and K. Hisatsune, J. Bacteriol., 85, 16, (1963).
6 - K. Takeya, K. Hisatsune and Y. Inoue, J. Bacteriol., 85, 24, (1963).
7 - A. Misaki, S. Yukawa, T. Tsuchiya and T. Yamasaki, J. Biochem., 59, 388, (1966).
8 - A. Misaki and S. Yukawa, J. Biochem., 59, 511, (1966).
9 - F. Kanetsuna, Biochim. Biophys. Acta, 158, 130, (1968).

10 - E. Vilkas and C. Nacasch, personal communication.
11 - I. Azuma and Y. Yamamura, J. Biochem., 53, 275, (1963).
12 - J.C. Ensign and R.S. Wolfe, J. Bacteriol., 91, 524, (1966).
13 - E.A. Hall and K.W. Knox, Biochem. J., 96, 310, (1965).
14 - N.P. Acharya, Thèse de Doctorat, Paris, 1967.
15 - J.M. Ghuysen, D.J. Tipper and J.L. Strominger in Methods in Enzymology VIII p. 685, Académic Press, New York, 1966.
16 - J.C. Ensign and R.S. Wolfe, J. Bacteriol., 90, 395, (1965).
17 - N. Sharon, T. Osawa, H. Flowers and R.W. Jeanloz, J. Biol. Chem., 241, 223, (1966).
18 - M. Leyh-Bouille, J.M. Ghuysen, D.J. Tipper and J.L. Strominger, Biochemistry, 5, 3079, (1966).
19 - D.W. Thomas, unpublished experiments.
20 - J. van Heijenoort, L. Elhaz, P. Dézelée, J.F. Petit, E. Bricas and J.M. Ghuysen, Biochemistry, 8, 207, (1969).